Towards an explanation for the success of *Acinetobacter baumannii* in the human host

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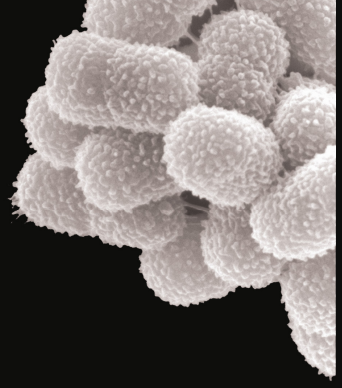

Chapter 1

General introduction and outline of the thesis

Introduction

Acinetobacter baumannii is a Gram-negative, non-motile bacterium notorious for its ability to colonize and spread among hospitalized patients. The fact that *A. baumannii* is more frequently associated with colonization than infection, even in susceptible patients, emphasizes the relatively low virulence of this bacterium. However, severe *A. baumannii* infections, including pneumonia and bacteremia, do occur in critically ill patients. Given the accumulating numbers of *A. baumannii* strains resistant to the last resort antibiotics, today's global nosocomial spread is a major cause of concern. It remains unclear which factors determine the clinical success of *A. baumannii*. The aim of the studies presented in this thesis was to obtain insight into the factors contributing to the epidemicity and pathogenicity of this bacterium. In this chapter, the current state of knowledge regarding *Acinetobacter* species, including their taxonomy, epidemiology, antibiotic resistance, clinical features and pathogenesis, is reviewed.

Taxonomy, epidemiology and antibiotic resistance

Bacteria belonging to the genus *Acinetobacter* have undergone significant taxonomic changes during the last decades. Until the mid 1980s, this genus comprised only a single species, *A. calcoaceticus* [1], although a second species, *A. lwoffii*, was included on the Approved Lists of Bacterial Names [2]. At the time, glucose-acidifying strains were also often referred to as '*Bacterium anitratum*' [3]. With the recent description of *A. pittii* [genomic species (gen. sp.) 3], *A. nosocomialis* (gen. sp. 13TU) [4], *A. rudis* [5] and *A. brisouii* [6], the genus *Acinetobacter* currently comprises 27 validly named species. The species *A. baumannii, A. calcoaceticus, A. pittii* and *A. nosocomialis* are genotypically closely related and phenotypically difficult to distinguish, which has given rise to the proposal to group them in the *A. calcoaceticus - A. baumannii* (Acb) complex [7]. From a clinical perspective this is not appropriate as the complex combines three clinically relevant species (*A. baumannii*, *A. pittii*, and *A. nosocomialis*) [8–12] with an environmental species (*A. calcoaceticus*) that is occasionally encountered in clinical specimens, but to our knowledge not associated with infection [13]. It is noteworthy that due to the difficulty in separating these Acb complex species with commercial identification systems commonly used in diagnostic microbiology [13,14], the clinical role of *A. pittii* and *A. nosocomialis* may be underestimated.

The ecology of bacteria belonging to the genus *Acinetobacter* is diverse. These organisms have been recovered from soil, surface water, vegetables, animals, human

body lice, and humans [15–19]. Twenty-five to 44% of healthy individuals were found to carry *Acinetobacter* species on the skin, particularly at moist sites like the axillae and toe webs [16,19]. The majority of the *Acinetobacter* species isolated from the skin belong to *A. lwoffii*, *A. johnsonii* and *A. junii*, whereas *A. baumannii* skin carriage is rare in healthy individuals [19]. The natural reservoirs of *A. baumannii* remain to be discovered. Bacteria of this species have been isolated mainly from hospitalized patients, but also from the hospital environment, and from sick animals [20]. There are indications that skin and mucous membranes colonized by clinically relevant species is an important source of infections in hospitalized patients, thereby contributing to the development and persistence of outbreaks [7,21,22]. *A. baumannii* has the capacity to survive on inanimate surfaces, such as ventilation equipment and bedding materials [23–27], for up to five months [28,29], thereby creating an important secondary reservoir from which spread to patients may occur.

The incidence of *A. baumannii* infections varies widely: from less than 1% in different European hospitals [30,31] to e.g. 32% among ventilated patients in a Taiwanese hospital [32]. *A. baumannii*, *A. pittii* and *A. nosocomialis* are the species most frequently involved in these infections [11,33–35]. *A. baumannii* strains have become endemic in multiple centres [36–40] and outbreaks have been observed worldwide [13,41]. A systematic review of published nosocomial outbreaks in the intensive care units (ICU) setting from 2005 to 2010 has revealed that *A. baumannii* was responsible for almost 25% of ICU infection outbreaks [42]. Three major lineages of genetically highly related *A. baumannii* strains, designated European (EU) clone I, II and III, are frequently implicated in outbreaks [43,44]. A recent striking manifestation is the occurrence of *A. baumannii* infections in soldiers severely injured during the conflicts in Iraq and Afghanistan [45]. Although *Acinetobacter* is mainly associated with nosocomial infection, several cases of community-acquired pneumonia, mostly associated with underlying diseases, have been reported [46–48].

During the last three decades, clinicians are increasingly faced with infections of *A. baumannii* isolates resistant to almost all clinically applicable antibiotics [12,49,50]. By the late 1990s, carbapenems and polymyxins were the only remaining useful agents that could combat severe infections with this microorganism in certain hospitals. To date, however, carbapenem-resistant strains are accumulating worldwide [12,49,51], a worrying development given the paucity of alternative treatment agents [52]. The ability of *A. baumannii* to persist in the hospital environment and to colonize patients in addition to its multidrug-resistant (MDR) phenotype makes control of outbreaks caused by this organism difficult [7,53].

Clinical manifestation and risk factors

Acinetobacter is an opportunistic pathogen that almost exclusively affects predisposed individuals, i.e., critically ill patients, including intensive care unit (ICU) and heavy trauma patients, as well as hospitalized neonates [7,54–56]. It can cause various types of infection, including pneumonia, urinary tract infections, skin and wound infections, and bacteremia [7,41]. These infections are mainly caused by *A. baumannii*, although *A. pittii* and *A. nosocomialis* also play a role [33,35,57–62]. Hospital-acquired infections caused by other *Acinetobacter* species are rare and if they occur, the clinical course is usually mild.

Prolonged hospital stay and previous antibiotic use have been identified as risk factors for *A. baumannii* infection [63–65]. In addition, invasive devices, such as tubes, catheters, and vascular devices may act as portes d'entrée for *Acinetobacter* species [64,65]. Consequently, *A. baumannii* has emerged as a particularly important organism in ICUs, responsible for increased length of hospital stay and consequently of healthcare costs [66,67].

Mortality in patients with *A. baumannii* infection in the ICU fluctuates widely, depending on patient characteristics, such as age and immune status [68], but also on geographic region; in the USA, crude mortality rates of 2% to 34% have been described [69–71], whereas in Taiwan and Turkey, rates as high as 20-68% [72–75] and 60-81% [76,77] have been reported, respectively. A multicenter study of *Acinetobacter* outbreaks in the Netherlands from 1999 to 2001 showed that in 1% of cases, death was a direct consequence of *A. baumannii* infection, whereas it had possibly played a role in the death of 8% of the infected patients [26]. The impact of *A. baumannii* on mortality remains a matter of debate as it is often difficult to determine whether mortality is caused by the patient's underlying illness or by the *A. baumannii* infection [7,78,79].

Altogether, MDR *A. baumannii* has a major clinical impact on the healthcare setting. In the next section, the factors that play a role in the pathogenesis of this troublesome pathogen will be discussed.

Pathogenesis and host defenses

The balance between on the one hand the host's defense and on the other particular bacterial characteristics, so-called virulence factors [80], determines the outcome of infection. In the following paragraphs, we will discuss host defense mechanisms in general and the current knowledge of the virulence factors of *A. baumannii* (summarized in Figure 1).

Host defense mechanisms

The first line of defense encountered by pathogens is the anatomical barrier presented by the skin and mucous membranes. Chemical features of these epithelia, including the presence of fatty acids, low pH, antimicrobial peptides and enzymes, help to prevent pathogen entry [81]. Moreover, a key component of mucosal secretions, lactoferrin, binds iron and thereby hinders bacterial growth. If pathogens do cross the protective epithelial barrier, humoral and cellular effectors of the host innate immune response come into play. The major humoral innate defense mechanism, the complement system, is activated in the presence of pathogens [82,83]. Activation leads to the deposition of C3b on the pathogen surface, initiating opsonisation for phagocytosis and the formation of the membrane-attack complex (MAC), which can cause direct lysis of the pathogen. The chemotactic activity of complement component C5a attracts immune cells from the circulation. Pathogens can also be recognized by specific pattern recognition receptors on host cells, including Toll-like receptor (TLR)2, TLR4, TLR5, and TLR9 and C-type lectins [84,85]. This results in the production and release of chemokines and cytokines and the subsequent recruitment of neutrophils and monocytes from the circulation. Neutrophils phagocytose and kill engulfed pathogens through the production of toxic reactive oxygen species (ROS) mediated by NADPH oxidase and myeloperoxidase as well as non-oxidative mechanisms. The recruited monocytes differentiate, depending on the local environment, into macrophages or dendritic cells that can phagocytose and kill the pathogen but are also involved in initiating an adaptive immune response. In response to bacteria, host cells can produce antimicrobial peptides, including the cathelicidin LL-37 and human defensins, that inhibit bacterial growth and modulate the host response [86]. Although these inflammatory reactions are essential to eradicate the pathogen, they can also contribute to a wide range of deleterious effects, such as tissue damage [87].

Many bacteria have evolved different mechanisms to resist or evade these host defenses, as described below and summarized in Figure 1.

Virulence mechanisms

Adherence

The ability of bacteria to adhere to surfaces is the initial step in the colonization of various niches [88,89]. *A. baumannii* can adhere to different abiotic surfaces, such as glass and plastic [90–96], for prolonged periods [97,98], which may contribute to its persistence on medical devices and the hospital environment [28].

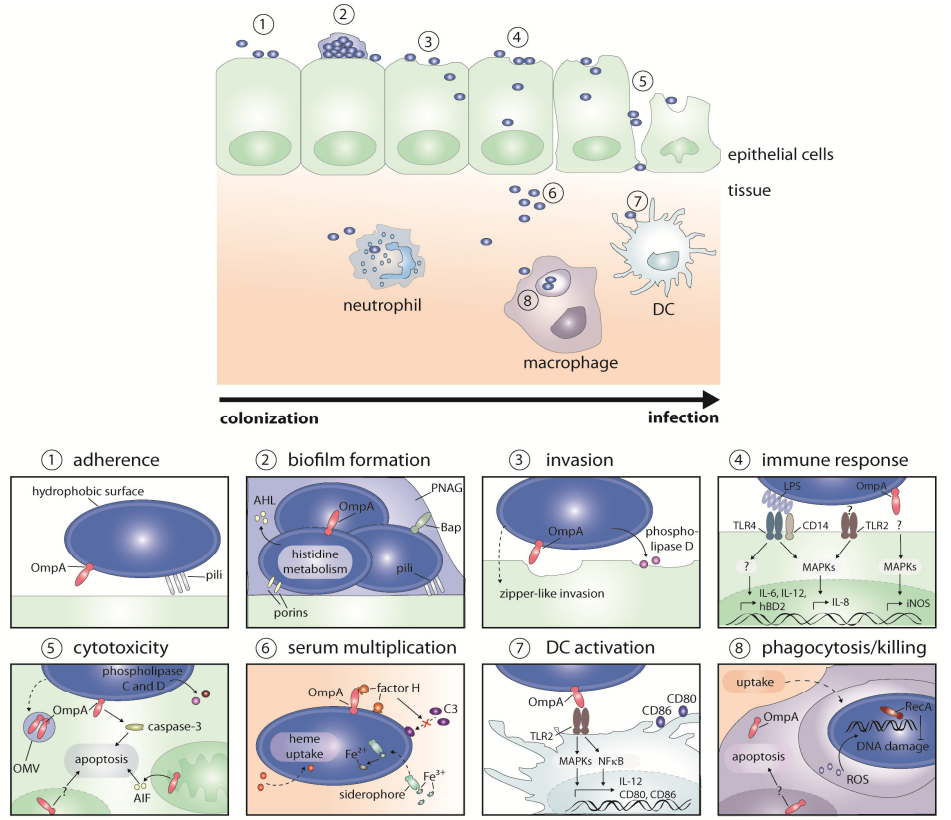

Figure 1. Overview of the interaction of *A. baumannii* with its host. Bacteria can adhere to the host surface (**1**) via adhesins and aspecific factors. They multiply, form microcolonies and produce exopolysaccharides resulting in the formation of a biofilm (**2**). Quorum sensing, histidine metabolism, specific outer membrane proteins and pili play a role in this process. Once adhered, bacteria can invade the host via a zipper-like mechanism or secrete several enzymes that degrade host cell molecules enabling them to enter the underlying tissues (**3**). Such toxic agents produced by the bacteria can, either directly or via outer membrane vesicles, be transferred to the host cells leading to apoptosis (**5**). The host cells can sense the bacteria via specific pattern recognition receptors, which triggers an innate immune response (**4**). Once inside the host, bacteria activate dendritic cells (**7**), which subsequently activate the adaptive immune response. Neutrophils and macrophages residing in the tissues or bloodstream can phagocytose and kill the invaded bacteria (**8**). However, bacteria can also prevent killing by these cells either by inducing apoptosis of the host cells or by preventing ROS-induced DNA damage. The ability of bacteria to evade the killing actions of the complement system, e.g., by binding complement inhibitors to their surface, and the ability to acquire iron via siderophores enables them to survive and multiplicate inside the host (**6**).
AHL, acylated homoserine lactones; AIF, apoptosis inducing factor; Bap, biofilm-associated protein; DC, dendritic cell; hBD, human beta defensin; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; NFκB, nuclear factor κB; Omp, outer membrane protein; OMV, outer membrane vesicle; PNAG, poly-β-(1-6)-N-acetyl glucosamine; ROS, reactive oxygen species; TLR, Toll-like receptor.

Acinetobacter is also able to colonize biotic surfaces, including human skin and mucosal surfaces [16,19], which was corroborated by laboratory studies demonstrating that *Acinetobacter* strains could adhere to human epithelial cells in vitro [91,94,99,100].

Specific factors, such as adhesins, and aspecific factors like surface hydrophobicity play an integral role in bacterial adherence [81,94,101]. Adhesins are bacterial components capable of binding to a large variety of surfaces, thereby mediating interactions between the bacterium and a surface. They can broadly be divided into fimbrial and afimbrial adhesins [102,103]. Fimbriae are short, hair-like bacterial appendages [104] that can be assembled by various pathways, including the chaperone-usher pathway. Via this pathway two proteins are produced, a periplasmic chaperone, which facilitates the folding of pilus subunits and an outer membrane usher that facilitates the dissociation of the subunits of the chaperone, exposing the subunit´s interactive surface and driving its assembly into the pilus [103]. Genome analyses revealed the existence of different fimbrial genes in *A. baumannii* [105–107]. In agreement with these findings, various types of fimbrial adhesins have been identified in *Acinetobacter* species, including bundle forming thin pili, which are associated with adherence to abiotic and biotic surfaces, and individual thick pili that are associated with twichting motility, i.e., the ability to move on solid surfaces [108], enabling colonization of adjacent surface areas [92,93,95]. Pili mediated by the CsuA/BABCDE chaperone-usher system have been identified as important structures to enable adherence to glass and plastic surfaces [95]. Another chaperone-usher system with a similarly organised pilus gene cluster, the *acuA* cluster, was identified in the environmental species *A. baylyi* [92]. Altogether, these studies indicate that *Acinetobacter* species express different pili that are associated with the adherence to abiotic and biotic surfaces.

In addition to fimbrial adhesins, many bacterial species express a family of cell-surface adhesins that specifically recognize and bind components of the extracellular matrix [109]. Koljalg et al demonstrated that different clinical *A. baumannii* isolates could adhere to the extracellular matrix constituents collagen, fibronectin, fibrinogen and vitronectin [110], but the microbial surface components recognizing these adhesive matrix molecules have not been identified yet. Outer membrane protein A (OmpA) is the most abundant surface protein of *A. baumannii* and has been shown to be required for adherence of *A. baumannii* ATCC19606^T to fungal filaments and alveolar epithelial cells [111]. Other factors like surface hydrophobicity play a role in the adherence of *Acinetobacter* to abiotic [91,94] and cellular surfaces [92]. Clinical *A. baumannii* strains were found to constitute higher hydrophobicity as compared to *A. baumannii* strains isolated from skin of healthy individuals [112]. Thus, in addition to pili, other cell surface adhesins and surface hydrophobicity of *Acinetobacter* strains are thought to play a role in

their adherence to host surfaces.

Biofilm formation

Once bacteria have adhered to a surface, they can multiply and form microcolonies, followed by the production of exopolysaccharides, resulting in a highly structured microbial community: the biofilm [113,114]. The biofilm confers several advantages to bacteria within this structure as compared to their free-living counterparts: (i) the biofilm is an efficient scavenging system for trapping and concentrating essential minerals and nutrients from the environment [115]; (ii) sessile bacteria are more resistant to the effects of antibiotics and to effector cells and molecules of the host defense system than organisms in suspension [113]; and (iii) the proximity of bacteria within the biofilm provides an ideal environment for the horizontal transfer of genes, including antibiotic resistance genes [116].

The ability of *A. baumannii* to form biofilms on glass and plastic surfaces [95,96,117] has been shown to depend on CsuA/BABCDE-mediated pili [95]. The expression of these pili is regulated by a two-component regulatory system comprised of a sensor kinase encoded by *bfmS* and a response regulator encoded by *bfmR* [118]. In addition, OmpA is needed for the formation of robust biofilms on abiotic surfaces [111]. Outer membrane proteins NlpE [106], Omp33 and the peptidoglycan-binding protein LysM as well as the putative porins CarO, OprD-like and DcaP-like proteins are also involved in *A. baumannii* biofilm formation on plastic, although their precise role in this process remains to be determined [119]. Choi et al described that the *pgaABCD* genes encode enzymes that synthesize the extracellular polysaccharide poly-β-(1-6)-N-acetyl glucosamine in *A. baumannii* [120], which is an important component of the biofilm. A homologue of the staphylococcal biofilm-associated protein (Bap) is involved in inter-bacterial adhesion in mature biofilms and is needed for the stabilization of *A. baumannii* biofilms formed on glass [121].

Biofilm formation is influenced by environmental factors, such as the concentration of free calcium and iron, both of which enhance *A. baumannii* biofilm formation on plastic [95,122]. Two recent studies described the involvement of proteins participating in histidine metabolism in biofilm formation by *A. baumannii*, although the precise role still needs to be identified [119,123]. In this connection, it was suggested that *A. baumannii* reprograms bacterial gene expression by using certain aminoacids like histidine, leading to biofilm formation as described for *Pseudomonas putida* [124].

The association between *A. baumannii* biofilm formation, antibiotic resistance and clinical success is ambiguous. Rodriguez-Bano et al [125] and King et al [126] did not find that MDR resistant *A. baumannii* isolates were more frequently biofilm-formers on

abiotic surfaces as compared to susceptible isolates, in contrast to several other studies [127,128]. It is of note that all these studies only examined an indirect association by comparing antibiotic resistant and susceptible strains for their ability to form a biofilm. Shin et al reported that the extended-spectrum β-lactamase PER-1 was up-regulated in *A. baumannii* cultured under biofilm conditions [123]. Another study has demonstrated a positive association between the expression of *blaPER-1* and the ability of *A. baumannii* to form a biofilm [122]. Furthermore, a putative resistance nodulation cell division type efflux pump (RND pump) is increased in bacteria residing in biofilms. Overexpression of this type of efflux pump in *A. baumannii* confers resistance to different antibiotics [129,130], suggesting that changes in the *A. baumannii* proteome during biofilm formation play a role in the resistance to antibiotics.

Altogether, *A. baumannii* in biofilm structures are protected against environmental stresses, such as desiccation [131] and exposure to antibiotics, which may explain the survival properties of this organism on environmental surfaces and medical devices.

Quorum sensing

In order to build a highly structured and complex biofilm community, bacteria are thought to communicate with one another through a process called quorum sensing [132]. The term 'quorum sensing' describes the ability of bacteria to monitor the microbial population density, which is reflected by levels of specific signal molecules, autoinducers [132]. In Gram-negative bacteria, quorum sensing is typically mediated by autoinducers belonging to the group of acylated homoserine lactone derivatives (AHLs) [133]. These signal molecules are synthesized at a low basal level and diffuse from the bacterium according to their concentration gradient. Increased population density results in the accumulation of AHLs, which interact with transcription factors that control the expression of, among others, virulence genes [134,135]. Smith *et al* recently described the existence of genes encoding autoinducer processing in a clinical *A. baumannii* strain [107]. This is supported by the finding of three different quorum sensing signal molecules among *Acinetobacter* species, including *A. baumannii*, *A. pittii* and *A. nosocomialis* [136,137] and several studies that described an association between autoinducer production and biofilm formation by *Acinetobacter* [138–140].

Invasion

Once adhered to the host surface, pathogens may gain access into the host by a process called invasion [141]. For this purpose, pathogens can secrete several enzymes that degrade host cell molecules enabling them to disseminate into the host while avoiding

uptake by host cells, so-called extracellular invasion. Intracellular invasion occurs when a pathogen actually penetrates host cells, which can occur via two mechanisms: a zipper-like mechanism, which requires the direct interaction of bacterial ligands to host's cell surface receptors; and a trigger mechanism, which is initiated by injection of bacterial effector proteins into host cells by a type III secretion system. In both cases, the host's cytoskeleton is rearranged to promote bacterial invasion.

A. baumannii can invade different respiratory epithelial cells [111,142] via a zipper-like mechanism [142]. OmpA plays a role in the invasion of these cells by *A. baumannii* ATCC19606^T [111,142] and was involved in the dissemination of this strain into the bloodstream in a mouse pneumonia model [142]. *A. baumannii* secretes enzymes that can potentially degrade host cell molecules, including lipases and hemolysins [97], by which this pathogen may invade the host. Moreover, Jacobs et al reported a role for phospholipase D of *A. baumannii* in bronchial epithelial cell invasion in vitro and dissemination in vivo [143]. These so-called invasins can, in addition to promoting bacterial invasion, also induce cytotoxicity as described below.

Induction inflammatory responses

The bacterial cell envelope-associated lipopolysaccharide (LPS) is considered to be the principal component of Gram-negative bacteria that is responsible for the induction of the host's immune response. The lipid A part of LPS of different *A. baumannii* isolates is a bisphosphorylated diglucosamine with at least six saturated fatty acyl chains with lengths of 12 or 14 carbons [163], a structure that is associated with a high cytokine-inducing capacity [164]. This notion is supported by different studies showing that LPS of *A. baumannii* and *A. hemolyticus* induced an inflammatory response in vivo [163,165,166]. TLR4 plays a key role in innate sensing of *A. baumannii* via the LPS moiety, in initiating an adequate inflammatory response and, hence, in effective elimination of bacteria from e.g. the lung and in prevention of systemic bacterial spread. TLR2, however, reduced inflammatory innate responses and delayed eradication of *A. baumannii* in a murine model of pneumonia [166]. In vitro, both TLR2 and TLR4 have been shown to engage with *A. baumannii* [163,167], triggering activation of NF-κB and mitogen-activated protein kinases (MAPKs) pathways and the subsequent production of IL-8 in alveolar epithelial cells [163]. OmpA is another immunomodulatory factor of *A. baumannii* [168]. It was found to be associated with the activation and maturation of dendritic cells that drive T cell differentiation towards a T helper cell type 1 response [168].

Only a few studies have investigated host's immune responses to *A. baumannii*. It was shown that C57BL/6 mice were capable of clearing *A. baumannii* within 72 h after intranasal inoculation with relatively high doses [166,169–171]. These results suggest that

innate immune defenses in the lungs can effectively control *A. baumannii* infection and prevent development of severe disease. In mice, *A. baumannii* induces the production of moderate amounts of the pro-inflammatory cytokines and chemokines tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6, IL-17, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-2 and keratinocyte chemoattractant (KC) [166,169–173], resulting in the recruitment of neutrophils. Early recruitment of neutrophils into the lung has been shown to be important for initiating an efficient host defense against respiratory *A. baumannii* infection in mice [169,171,172].

Neutrophils can engulf microbes into a phagosome that fuses with intracellular granules to form a phagolysosome, in which bacteria are killed through the action of reactive oxygen species (ROS) and oxygen-independent mechanisms such as antimicrobial peptides [174]. Qiu et al demonstrated a crucial role for NADPH oxidase-dependent ROS in the neutrophil-mediated killing of *A. baumannii* ATCC17961 in a mouse model of pneumonia [175]. However, *A. baumannii* may also evade killing after phagocytosis as has been described for a strain that induced the expression of several oxidative-stress responsive proteins during the late stationary phase of growth in vitro, which may function in ROS detoxification [176]. Furthermore, another study described the role of the enzyme RecA in protection of *A. baumannii* from DNA damaging agents, including ROS produced by macrophages in vitro [177]. In addition to the oxidative-dependent killing mechanisms, *Acinetobacter* has also been shown to be susceptible to the antimicrobial peptides present in neutrophils [178,179]. Different *A. baumannii* strains are susceptible to the antimicrobial action of human beta defensin 2, which is produced by alveolar epithelial cells in response to this pathogen, showing that epithelial cells are capable of producing mediators necessary for the clearance of *A. baumannii* [163].

Cytotoxicity

The ability to produce toxins plays a crucial role in the pathogenesis of many bacteria [144]. Toxic agents can be secreted by the pathogen into the surrounding environment or directly injected into the host cell via e.g. a type III secretion system. Although *A. baumannii* has not been documented to produce specific toxins, strains of this species have been shown to be toxic to human respiratory epithelial cells and macrophages [142,145–147]. OmpA was involved in the induction of cell death in these cells [145] as well as in fungal filaments [111] by activating caspase-dependent and apoptosis inducing factor (AIF)-dependent pathways [145]. In addition, OmpA was shown to be present in outer membrane vesicles (OMVs) produced by a clinical *A. baumannii* isolate [148] that bound to host cells [149]. It was postulated that OMVs from *A. baumannii* were thus a vehicle to deliver OmpA and probably other effector molecules into host cells.

Iron acquisition

Iron is a vital nutrient for virtually all forms of life as it has an essential role in many cellular processes. The vast majority of iron in the vertebrate host is bound to the iron storage proteins ferritin, transferrin and lactoferrin or complexed within the porphyrin ring of heme, making the levels of free iron in the tissues and bloodstream very low [150]. One of the most commonly found strategies evolved by microorganisms to survive in these iron-limited conditions of the host is the synthesis and excretion of low molecular weight compounds with a very high affinity for iron, called siderophores [151–153]. These siderophores may compete directly with host proteins for bound iron in order to make it available for the microbial cell [152]. Bacteria may also access iron through Omp receptors that recognize the whole complex of host proteins and iron, allowing the internalization of iron without the intermediacy of a siderophore [151].

Several studies have shown that *A. baumannii* isolates have the ability to grow under iron-limiting conditions in vitro [97,154–156] and different siderophores, heme-acquisition and ferrous iron-uptake systems, have been described [97,154,155,157–160]. Furthermore, inner and outer membrane proteins related to FatA, which is part of an outer membrane receptor that recognizes and internalizes iron-siderophore complexes in the fish pathogen *Vibrio anguillarum* [161], were detected in a clinical isolate of *A. baumannii* [157]. The wide distribution of multiple iron-acquisition systems among different *A. baumannii* isolates suggests that iron-acquisition is an indispensible attribute, and hence, an important virulence factor.

Serum multiplication

When invading the tissues and the bloodstream, bacteria have to evade the killing actions of the complement system in order to survive. Some *A. baumannii* strains are shown to be resistant to the killing action of human serum [97,126,143,162]. OmpA plays a role in serum resistance of *A. baumannii* by binding complement factor H, thereby inhibiting the alternative complement pathway [162]. It was also found that the production of phospholipase D is associated with serum resistance of *A. baumannii* [143], although the mechanism involved has not been characterized yet. The resistance of *A. baumannii* to the killing actions of complement likely plays an important role in the ability of the organism to survive in the host's tissues and bloodstream.

Outline of the thesis

Altogether, a variety of virulence factors have been identified in *A. baumannii*. However, in most studies only one or a few strains have been used and it is not known whether these findings can be extrapolated to other strains of this species. Moreover, not all studies have used well-validated genotypic species identification methods, making it difficult to extrapolate findings to particular species. Whether these factors explain the clinical success of *A. baumannii* relative to other *Acinetobacter* species remains to be determined. The studies presented in this thesis aimed to improve insight into the bacterial and host factors associated with the pathogenesis of *A. baumannii* as a first step towards an explanation for the success of *A. baumannii* as a human pathogen.

The three main questions underlying the studies presented in this thesis were:

(i) Can adherence to and biofilm formation on (a)biotic surfaces explain the clinical success of *A. baumannii* relative to other *Acinetobacter* species?

(ii) What is the role of the host innate immune response in the clinical success of *A. baumannii* relative to other *Acinetobacter* species?

(iii) Which virulence attributes are expressed by *A. baumannii*?

To answer these questions, we made use of a large collection of epidemiologically well-described strains that has been set up during an ongoing project at the Leiden University Medical Centre. This project was started up in the early 1980s at the Dijkzigt Rotterdam University Hospital (currently the Erasmus University Hospital Rotterdam) and focuses on the epidemiology and taxonomy of *Acinetobacter*. We selected strains that differ in their behaviour 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, clinically less-relevant *Acinetobacter* species. In **chapter 2**, we compared these strains and species for their ability to form biofilms on abiotic surfaces, to adhere to human cells and to induce an innate immune response in these cells. In **chapter 3**, we studied the role of CsuA/BABCDE-dependent pili, which have been described to be involved in biofilm formation on abiotic surfaces [95] in the adherence to human cells and in the induction of an inflammatory response in these cells. To investigate the role of the host response in more detail, we compared the persistence of and host response to different well-characterized *A. baumannii* strains and an *A. junii* strain in a mouse pneumonia model (**chapter 4**). We used a systems biology approach involving genomics, phenomic and virulence studies in order to investigate the genetic basis of the different virulence attributes and to identify species characteristics that may explain why some *Acinetobacter*

species are successful as human pathogens and others are not (**chapter 5**). Several papers have suggested that membrane vesicles play a role in the delivery of virulence factors to host cells [148,149] but it is not clear how and when in the life cycle of the bacterium these vesicles are produced. Moreover, it is not known whether laboratory procedures influenced the generation of particular vesicles. We used advanced electron microscopy techniques in combination with a lenient vesicle purification method to investigate the formation and structure of membrane vesicles produced by *A. baumannii* in different growth phases (**chapter 6**). As a novel model to study virulence attributes, we developed a three-dimensional human epidermal skin model of *Acinetobacter* colonization (**chapter 7**). Finally, the main findings from our studies are summarized and discussed in **chapter 8** and a summary in Dutch can be found in **chapter 9**.

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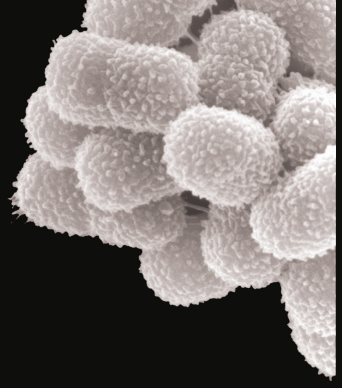

Chapter 2

Do biofilm formation and interactions with human cells explain the clinical success of *Acinetobacter baumannii*?

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Abstract

The dramatic increase in antibiotic resistance and the recent manifestation in war trauma patients underscore the threat of *Acinetobacter baumannii* as a nosocomial pathogen. Despite numerous reports documenting its epidemicity, little is known about the pathogenicity of *A. baumannii*. The aim of this study was to obtain insight into the factors that might explain the clinical success of *A. baumannii*.

We compared biofilm formation, adherence to and inflammatory cytokine induction by human cells for a large panel of well-described strains of *A. baumannii* and compared these features to that of other, clinically less relevant *Acinetobacter* species. Results revealed that biofilm formation and adherence to airway epithelial cells varied widely within the various species, but did not differ among the species. However, airway epithelial cells and cultured human macrophages produced significantly less inflammatory cytokines upon exposure to *A. baumannii* strains than to strains of *A. junii*, a species infrequently causing infection.

The induction of a weak inflammatory response may provide a clue to the persistence of *A. baumannii* in patients.

Introduction

With the recent description of *Acinetobacter bereziniae* (genomic species (gen. sp.) 10), *A. guillouiae* (gen. sp. 11) [1], *A. venetianus* [2] and *A. soli* [3], the genus *Acinetobacter* comprises 23 named species and 11 species with provisional designations. Of these, *A. baumannii* and the closely related *A.* gen. sp. 3 and 13TU are clinically the most relevant. Strains of these species have the ability to colonize and spread among critically ill hospitalized patients. Outbreaks of multidrug resistant *A. baumannii* strains have been observed worldwide [4], [5]. A striking manifestation is the dramatic increase in the frequency of imipenem resistant *Acinetobacter* isolates in US hospitals [6] and the recent occurrence of infection in severely injured soldiers during the conflicts in Iraq and Afghanistan [7]. Three major lineages of genetically highly related *A. baumannii* strains, designated European clone I, II and III, have been found to be frequently implicated in outbreaks [8], [9]. Other *Acinetobacter* species, such as *A. junii*, *A. johnsonii* and *A. lwoffii* that can frequently be found on the human skin are only incidentally involved in infection, which usually has a mild course [4]. This suggests differences in the pathogenic potential among *Acinetobacter* species.

The high prevalence of *A. baumannii* strains in the hospital in epidemic and endemic situations might be explained by several factors, including their resistance to antibiotics [10] and desiccation [11], their ability to form biofilms on medical devices [12], and to colonize skin and mucosal surfaces of vulnerable hosts [13], [14]. Adherence of bacteria to host cells is generally considered to be an essential initial step in the colonization process [15]. Once the primary colonizing bacteria have attached to a surface, microcolonies are formed after which bacteria may secrete exopolysaccharides resulting in a highly structured sessile microbial community, the biofilm [16]. Several studies have documented the ability of *A. baumannii* to adhere to epithelial cells and to form biofilms on glass and plastic surfaces [12], [17], [18]. Adherent bacteria can interact with cells of the host defense systems resulting in the release of cellular mediators and effector molecules, such as interleukin (IL)-6 and IL-8 and antimicrobial peptides, which help to eradicate the pathogen [19].

Little is known about the pathogenicity of *A. baumannii*. Moreover, the scarce reports on the virulence of *A. baumannii* are focused on one or a few strains only. The purpose of the present study was to obtain insight into the factors that might explain the clinical success of *A. baumannii*. To this aim, biofilm formation was investigated for a large set of well-described *A. baumannii* strains that differed in epidemicity and clonality. Next, biofilm formation by *A. baumannii* was compared to that of other *Acinetobacter* species, including *A.* gen. sp. 3 and 13TU, *A. calcoaceticus* and *A. junii*. For a subset of *A. baumannii*

and *A. junii* strains, adherence to airway epithelial cells and induction of inflammatory cytokine production by these cells and cultured human macrophages was quantitated. Furthermore, the presence of pilus-like structures that may play a role in adherence and biofilm formation was assessed with scanning electron microscopy (SEM).

Materials and Methods

Bacterial strains, culture conditions and antimicrobial susceptibility testing

Forty-five *A. baumannii*, 3 *A.* gen. sp. 3, 3 *A.* gen. sp. 13TU, 3 *A. calcoaceticus*, and 7 *A. junii* isolates, were selected from the Leiden University Hospital *Acinetobacter* collection for this study. Of the *A. baumannii* strains, 18 were from outbreaks, 16 presumably not from outbreaks on basis of time-space-origin, and 11 of which the association with an outbreak was unknown. Eight of the *A. baumannii* strains belonged to European clone I, 11 to clone II, and 3 to clone III. All isolates had previously been identified to species by one or more validated genotypic identification methods [27], [28], [29]. Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at -80°C. Prior to each experiment, inocula from frozen cultures were grown overnight at 30°C [30] on sheep blood agar plates (BioMerieux, Boxtel, the Netherlands). For experiments, fresh subcultures were made either under these conditions or in Luria-Bertani (LB) medium. Susceptibility to antimicrobial agents was determined by disc diffusion according to CLSI recommendations [31]. Strains resistant to more than two of the following drug classes were defined as multidrug resistant: cephalosporins, carbapenems, ampicillin-sulbactam, quinolones, aminoglycosides.

Biofilm formation

Biofilm formation in 96-wells polyvinylchloride microtiter plates (Falcon, BD, Breda, the Netherlands) was assayed as described [32]. Briefly, bacteria from an overnight culture in LB medium were suspended to 1×10^6 colony forming units (CFU)/ml as calculated from the absorbance of a suspension at 600 nm. Five µl of this suspension was inoculated in 100 µl of M63 medium consisting of KH_2PO_4 (12 g/l), K_2HPO_4 (7 g/l), $(NH_4)_2SO_4$ (2 g/l), glucose (0.2% w/v), $MgSO_4$ (1 mM) and casaminoacids (0.5% w/v). After 24 h incubation at 28°C and 37°C, wells were washed and biofilms attached to the wells were stained with crystal violet (1% w/v). The optical density at 590 nm, expressed in arbitrary units (a.u.), was taken as a quantitative measure of biofilm mass. To determine the bacterial concentration after 24 h, serial dilutions of the supernatants were made in phosphate buffered saline (PBS; pH 7.4) and plated onto blood agar.

Adherence to human airway epithelial cells

Adherence of bacteria to human bronchial epithelial H_{292} cells (ATCC CRL-1848, Manassas, VA, USA) was determined as described [18]. Briefly, H_{292} cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 µg/ml streptomycin, 1000 U sodium penicillin G, and 10% (v/v) heat-inactivated foetal calf serum (FCSi) (all from Gibco, Invitrogen, Breda, The Netherlands), further referred to as culture medium, in 25 cm^2 tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37°C/5% CO_2. At confluency, cells were trypsinized and $2x10^5$ cells were cultured on plastic coverslips (Thermanox, Nunc, Amsterdam, the Netherlands) placed in 24-wells plates. At 85-90% confluency, H_{292} cells were washed with PBS and incubated at 37°C/5% CO_2 in culture medium without antibiotics for 24 h. Subsequently, cells were washed with PBS and incubated for 1 h at 37°C with 1×10^7 (range 7×10^6–4×10^7) CFU of an overnight culture on blood agar. Bacterial adherence to H_{292} cells was quantified by light microscopy using two parameters: (i) percentage of epithelial cells associated with at least one bacterium; and (ii) average number of bacteria per epithelial cell [18].

Cytokine induction in human airway epithelial cells

Cytokine production by airway epithelial cells in response to bacteria was determined as described [33] with minor modifications. In short, bacteria were cultured overnight at 30°C on blood agar and suspended in RPMI-1640 to a concentration of 5×10^8 CFU/ml as assessed spectrophotometrically. Approximately 2×10^5 H_{292} cells, cultured in 24-wells plates as descrived above, were incubated in culture medium without antibiotics and FCSi for 24 h. Next, cells were washed with PBS and incubated for 1 h at 37°C/5% CO_2 with 1×10^6, 1×10^7, or 1×10^8 CFU live or heat-inactivated (by 1 h incubation at 100°C) bacteria. H_{292} cells were washed five times with PBS to remove non-adherent bacteria and RPMI-1640 with 2 mM L-glutamine was added. After additional 5 or 23 h incubation at 37°C, supernatants were collected and stored at −20°C. After 24 h, bacterial CFU count in the supernatants was determined. In each experiment, RPMI-1640 with 2 mM L-glutamine alone was added to the cells to determine background values. A mixture of cytokines (100 ng/ml TNFα, 20 ng/ml IL-1β and 10 ng/ml IFNγ; all from Biosource, Nivelles, Belgium) with 10 ng/ml rough type lipopolysaccharide (LPS, *Escherichia coli* J5, Sigma-Aldrich, Zwijndrecht, the Netherlands), further referred to as cytomix, was added to the cells as a positive control.

Cytokine induction in cultured human macrophages

Buffy coats from healthy human donors were purchased from Sanquin bloodbank, Amsterdam, the Netherlands, upon written consent with regard to scientific use. The

current study did not require approval from an ethics committee according to the Dutch Medical Research Involving Human Subjects Act. Monocytes were isolated from buffycoats by Ficoll amidotrizoate density centrifugation and magnetic sorting using anti-CD14-coated beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. Monocytes were resuspended in culture medium and cultured at a concentration of 2×10^5 cells/ml in wells of a 24-wells plate at 37°C/5% CO_2 in the presence of either 5 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (Biosource International, Camarillo, CA, USA) to induce differentiation of monocytes into macrophage type 1 or in the presence of 50 ng/ml recombinant human macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) for type 2 macrophages [20]. After three days, 0.5 ml of fresh culture medium was added. On the sixth day following isolation, cells were washed once in PBS and 0.5 ml RPMI-1640 with 2 mM L-glutamine was added. Bacteria were cultured overnight at 30°C on blood agar and suspended in PBS to a concentration of 5×10^7 CFU/ml as assessed spectrophotometrically. Cells were stimulated with 1×10^7 CFU of live bacteria for 24 h, after which supernatants were collected and stored at −20°C. In each experiment, PBS and LPS (100 ng/ml) were added to the cells as negative and positive control, respectively.

Determination of levels of inflammatory mediators

The levels of IL-6, IL-8, TNFα, IL-12p40 and IL-10 in culture supernatants were determined by ELISA (Biosource) according to the manufacturer's instructions. The lower limit of detection was 15 pg/ml for IL-6, 7 pg/ml for IL-8, and 25 pg/ml for TNFα, IL-12p40 and IL-10.

Electron microscopy analysis of bacterial surface structures

Bacteria from an overnight culture on blood agar at 30°C were suspended in PBS and fixed for 1 h at room temperature with 1.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (1:1). Fixed bacteria were transferred to poly-L-lysine-coated glass slides. After 1 h incubation at room temperature, bacteria were fixed to the slides with 1.5% glutaraldehyde for 30 min at room temperature. Thereafter, slides were washed twice in PBS, dehydrated through a series of graded ethanol, critical-point-dried, and coated with a layer of palladium-gold. Bacteria were examined using a JEOL JSM-6700F field emission scanning electron microscope.

Statistical analysis

Results are expressed as medians and ranges unless stated otherwise. The Kruskal-Wallis one-way analysis of variance and the Wilcoxon rank sum test were used to evaluate

differences in distribution. Spearman rank correlation coefficients were calculated to evaluate possible associations between epidemicity and adherence to epithelial cells and biofilm formation on plastic. P values of ≤0.05 were considered significant.

Results

Biofilm formation

Biofilm formation on plastic at 28°C and 37°C was first investigated for a comprehensive set of *A. baumannii* strains. The results revealed a large variation in biofilm formation among *A. baumannii* isolates; the results at 28°C and 37°C did not differ (Fig. 1). There was no difference in the median biofilm size between strains from outbreaks (0.9; 0–1.8 a.u.) and those not assumed to be from outbreaks (0.8; 0.1–2.8 a.u.). Strains of European clone II (1.1; 0.6–1.8 a.u.) formed larger (p≤0.05) biofilms than strains of clone I (0.8; 0–1.0 a.u.), but not larger than strains of clone III (1.0; 0.6–1.3 a.u.). Multidrug resistant strains (0.8; 0–1.8 a.u.) did not form larger biofilms than susceptible strains (0.8; 0.1–2.8 a.u.). Furthermore, no association between biofilm formation and body site of isolation was found (data not shown).

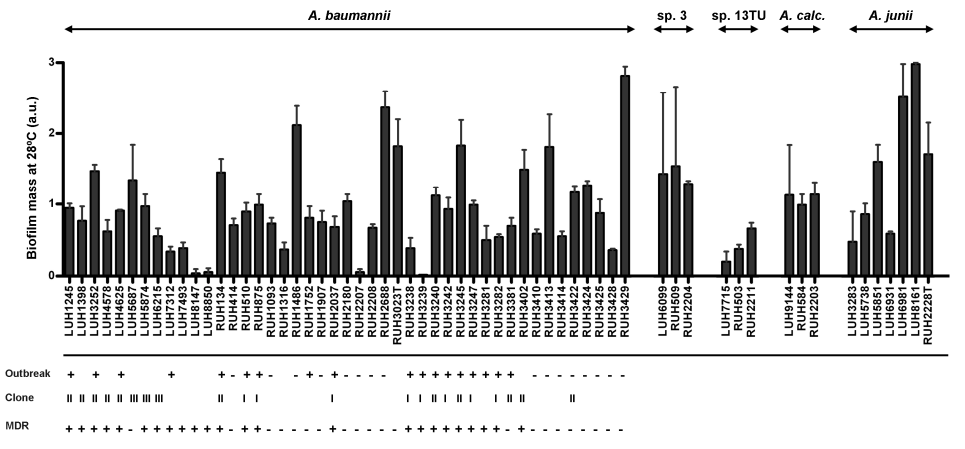

Figure 1. Biofilm formation by *Acinetobacter*. Biofilm formation after 24 h at 28°C for the clinically relevant *A. baumannii* (n=45), *A.* gen. sp. 3 (n=3) and *A.* gen. sp. 13TU (n=3) and for the clinically less-relevant *A. calcoaceticus* (n=3) and *A. junii* (n=7). Data are expressed as mean biofilm mass (in arbitrary units (a.u.)) of three independent experiments; each performed in sixplicate. Outbreak-associated (+) or non-outbreak-associated (−) isolate. European clone I (I), II (II) or III (III) isolate. Multidrug resistant (MDR; +) or susceptible (−) isolate.

Next, biofilm formation by *A. baumannii* strains was compared to that by strains of other *Acinetobacter* species. No significant differences in the median size of the biofilms at 28°C were found between clinically relevant species, i.e., *A. baumannii* (0.8; 0–2.8 a.u.), *A.* gen. sp. 3 (1.4; 1.3–1.5 a.u.) and *A.* gen. sp. 13TU (0.4; 0.2–0.7 a.u.), and the other *Acinetobacter* species, i.e., *A. calcoaceticus* (1.1; 1.0–1.1 a.u.) and *A. junii* (1.6; 0.5–3.0 a.u.). Similar results were obtained at 37°C except for strains of *A.* gen. sp. 13TU that formed smaller ($p \leq 0.05$) biofilms than all other species. Of note, the number of bacteria in the supernatants at the end of each experiment did not differ much between the different strains (data not shown).

Adherence to human airway epithelial cells

For further comparison of clinically relevant to less-relevant *Acinetobacter* strains and species, experiments exploring the ability of strains to adhere to and induce cytokines by human cells were conducted with a subset of strains (further referred to as subset), including six strains of *A. baumannii* and six strains of *A. junii*, a species infrequently causing infections (Table 1A). All selected strains were epidemiologically unrelated and were genotypically diverse as assessed by AFLP genomic fingerprinting.

Table 1A. Characteristics of subset of *Acinetobacter* strains

Strain	Origin		Year	Specimen	Clone	Outbreak‡
A. baumannii						
LUH1398	St. Zagora	(BG)	1993	Throat secr.	II	
LUH7312	Leiden	(NL)	2001	Sputum		+
LUH7493	Leiden	(NL)	2001	Sputum		
RUH875	Dordrecht	(NL)	1984	Urine	I	+
RUH3023T (ATCC19606)	Atlanta	(USA)	1965	Urine		
RUH3239	London	(UK)	1985	Urine	I	+
A. junii						
LUH3283	Leiden	(NL)	1995	Blood		
LUH5851	Leiden	(NL)	1999	Ear		
LUH6931	Nottingham	(UK)	2000	Faeces		
LUH6981	Leiden	(NL)	2000	Faeces		
LUH8161	Leiden	(NL)	2002	Blood		
RUH2228T (ATCC17908)	Heidelberg	(GER)	<1962	Urine		

‡Outbreak-associated (+) strain, (i.e. common AFLP profile in >2 patients and with same time-space-origin).

Adherence to human airway epithelial H_{292} cells varied widely among the *Acinetobacter* strains (Table 1B). No significant difference in the percentage of H_{292} cells associated with bacteria was observed between *A. baumannii* (30; 7–55%) and *A. junii* (23; 11–70%). In addition, no difference in the number of *A. baumannii* and *A. junii* per positive H_{292} cell was seen; the median number of bacteria per H_{292} cell was 3 (range 1–5). Of note,

differences in the inoculum (within the set range of 7×10^6–4×10^7) between the various experiments did not influence the outcome of the adherence assay. Furthermore, cell monolayers remained intact and the morphology of the cells was not affected by the bacteria (data not shown).

Table 1B. Biofilm formation, adherence and cytokine induction by *Acinetobacter*

Strain	Biofilm formation†		% Bacterial-associated cells‡		No. of bacteria per cell‡		IL-6 (ng/ml)¶		IL-8 (ng/ml)¶	
A. baumannii										
LUH1398	0.8	(0.5-1.1)	38	(32-65)	3	(3-3)	0.9	(0.0-3.9)	1.5	(0.4-4.8)
LUH7312	0.3	(0.3-0.4)	53	(38-76)	5	(3-6)	2.1	(0.1-14.5)	3.5	(0.8-12.8)
LUH7493	0.4	(0.3-0.5)	35	(16-46)	2	(2-3)	1.1	(0.0-3.6)	2.3	(0.1-4.8)
RUH875	1.0	(0.8-1.1)	24	(17-45)	3	(2-3)	1.2	(0.0-5.6)	2.0	(0.3-4.6)
RUH3023T (ATCC19606)	1.9	(0.9-2.2)	19	(9-21)	2	(1-2)	0.6	(0.0-2.7)	1.0	(0.1-3.5)
RUH3239	0	(0.0-0.0)	6	(3-14)	1	(1-2)	1.0	(0.0-4.4)	1.5	(0.1-3.5)
A. junii										
LUH3283	0.7	(0.0-0.8)	70	(62-83)	3	(3-4)	2.0	(0.1-11.9)	2.5	(0.8-10.0)
LUH5851	1.6	(1.4-1.8)	31	(15-41)	2	(2-3)	2.0	(0.2-11.8)	3.3	(1.6-8.1)
LUH6931	0.6	(0.6-0.6)	12	(9-13)	2	(2-2)	1.4	(0.0-7.4)	2.1	(0.4-8.7)
LUH6981	2.7	(2.6-3.0)	9	(5-29)	2	(1-2)	1.7	(0.1-12.3)	4.9	(1.5-11.5)
LUH8161	3.0	(3.0-3.0)	15	(8-25)	3	(3-3)	3.1	(0.1-8.7)	3.8	(0.7-9.9)
RUH2228T (ATCC17908)	1.7	(1.1-1.8)	47	(45-48)	3	(2-3)	2.2	(0.1-13.1)	3.0	(0.8-9.4)

†Biofilm formation on plastic after 24 h at 28°C. Results are expressed as median optical density values at 590 nm (ranges) of three independent experiments; each performed in sixplicate. ‡Adherence to human bronchial epithelial H_{292} cells after 1h. Results are expressed as median percentage of H_{292} cells that is associated with bacteria and median number of bacteria per H_{292} cells (range) of two independent experiments; each performed in duplicate. ¶IL-6 and IL-8 levels in the culture supernatants of H_{292} cells, 24 h after exposure to bacterial strains. Results are expressed as median values (range) in ng/ml of three independent experiments; each performed in triplicate.

Cytokine production by human airway epithelial cells in response to *Acinetobacter*

Pilot experiments demonstrated that 1×10^8 CFU bacteria induced higher ($p\leq0.05$) levels of the major cytokines IL-6 and IL-8 in H_{292} cells than 1×10^6 and 1×10^7 CFU did. Furthermore, stimulation with live bacteria resulted in higher ($p\leq0.05$) IL-6 and IL-8 production by H_{292} cells than heat-inactivated bacteria did. Time-course experiments demonstrated that the levels of IL-6 and IL-8 were higher ($p\leq0.05$) after 24 h than after 6 h stimulation. Therefore, further stimulation experiments were performed for 24 h with 1×10^8 CFU live bacteria.

Results revealed that H_{292} cells produced less ($p\leq0.05$) IL-8 in response to *A. baumannii* strains (1.8; 1.0–3.5 ng/ml) than to *A. junii* strains (3.2; 2.1–4.9 ng/ml; Fig. 2). Interleukin-6 production was lower in response to *A. baumannii* strains (1.1; 0.6–2.1 ng/ml) than to strains of *A. junii* (2.0; 1.4–3.1 ng/ml; Fig. 2), without reaching statistical significance ($p=0.055$). Of note, H_{292} cells stimulated with cytomix produced 2.3 (0.6–3.0) ng/ml IL-6 and 3.4 (1.4–4.1) ng/ml IL-8 and unstimulated cells produced 0.1 (0–0.1) ng/ml IL-6 and 0.2 (0.1–0.2) ng/ml IL-8. Cytokine induction did not vary widely among strains of

each species, except for *A. baumannii* strain LUH7312 that induced significantly higher levels of IL-6 and IL-8 than the other *A. baumannii* strains. Stimulation of H_{292} cells with 7 additional *A. baumannii* strains that were epidemiologically unrelated and genotypically diverse also resulted in low IL-8 (1.3; 0.3–1.7 ng/ml) and IL-6 production (0.1; 0–0.2 ng/ml), demonstrating that, with the exception of strain LUH7312, *A. baumannii* strains induced significantly ($p \leq 0.01$) less IL-6 and IL-8 than *A. junii* strains.

Since live bacteria were used in this assay, there was a possibility of outgrowth that may have caused the difference in cytokine induction. However, the number of bacteria in the supernatants at the end of each experiment did not differ much between *A. baumannii* and *A. junii* (range 8×10^6–7×10^7 CFU/ml). Of note, cell monolayers remained intact and the morphology of the cells was not affected by the bacteria (data not shown).

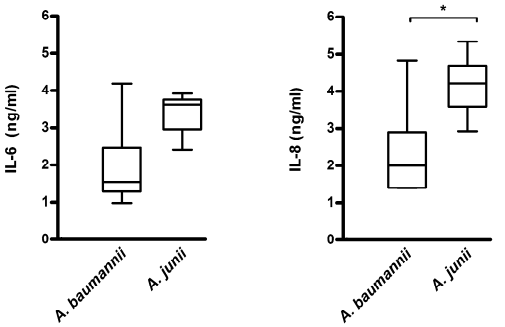

Figure 2. Cytokine production by human airway epithelial cells in response to *Acinetobacter*. Boxplots showing IL-6 and IL-8 production in ng/ml by H_{292} airway epithelial cells 24 h after stimulation with strains of *A. baumannii* (n=6) and *A. junii* (n=6). Boxes represent medians and second and third interquartiles, whiskers represent range of 6 strains. *significant ($p \leq 0.05$) difference between *A. baumannii* and *A. junii*.

Finally, preliminary experiments demonstrated that exposure of primary human bronchial epithelial cells of a single donor to six *A. baumannii* strains (in triplicate) resulted in lower ($p \leq 0.05$) levels of IL-8 (0.9; 0.5–3.6 ng/ml) than to six *A. junii* strains (4.5; 0.9–30.7 ng/ml). Cytomix induced 8.6 (7.4–11.5) ng/ml IL-8 in these cells. Of note, no IL-6 was produced by these primary cells upon exposure to these two *Acinetobacter* species or to cytomix.

Cytokine production by cultured human macrophages in response to *Acinetobacter*

In tissues, macrophages trigger an adequate innate immune response upon encountering pathogens. In this regulatory process, macrophages serve a dual purpose. Initially, they

contribute to the elimination of pathogens and the elicitation of an inflammatory reaction. When the infection recedes due to removal of the pathogens, their function may shift toward resolution of inflammation and tissue repair. In line with this notion, we investigated cytokine production by two clearly distinct types of macrophages, i.e., pro-inflammatory macrophages (further referred to as type 1 macrophages), and macrophages with an anti-inflammatory/pro-angiogenic phenotype (type 2 macrophages) [20], upon exposure to *A. baumannii* and *A. junii* strains. Results revealed that macrophage type 1 produced significantly ($p \leq 0.05$) less tumor necrosis factor (TNF)α, IL-12p40, IL-10 and IL-8 in response to strains of *A. baumannii* [195 (108–244) ng/ml TNFα, 7 (2-11) ng/ml IL-12p40, 22 (0-149) pg/ml IL-10 and 49 (27-66) ng/ml IL-8] than to strains of *A. junii* [650 (458–812) ng/ml TNFα, 130 (111–155) ng/ml IL-12p40, 764 (126–1587) pg/ml IL-10 and 111 (48–208) ng/ml IL-8; Fig. 3]. *A. baumannii* strains also induced less ($p \leq 0.05$) inflammatory cytokines in macrophage type 2 than *A. junii* strains (Fig. 3). Of note, cell monolayers remained intact and the morphology of the cells was not affected by the bacteria (data not shown).

Electron microscopy analysis of bacterial surface structures

Pili have been described to be involved in biofilm formation, adherence and the induction of an immune response [21]. Therefore, we performed SEM to assess the presence of such surface structures on four *A. baumannii* strains that differed in their ability to form biofilm and adhere to human cells. SEM of bacteria cultured for 16 h at 37°C on blood agar plates revealed two types of cell appendages: short pilus-like structures and long extensions (Fig. 4). The latter varied in length, were irregularly distributed over the cell surface, and sometimes connected bacteria. The pilus-like structures were detected in *A. baumannii* strain LUH1398, a large biofilm former and highly adherent, in LUH7312, a small biofilm former and highly adherent, and RUH3023^T, a large biofilm former but poorly adherent (Fig. 4, white arrows). Long cell extensions were seen in *A. baumannii* strain RUH3023^T and RUH3239, a small biofilm former and poorly adherent (Fig. 4, black arrows). In addition to these structures, there was a marked surface heterogeneity, from smooth (LUH7312) to pockmarked (LUH1398).

In contrast to *A. baumannii* strain RUH3023^T and RUH3239, strain LUH1398 and LUH7312 formed only a few cell clusters with no more than four cells grouped together on the glass slides (data not shown). No difference in structural features was observed between strains cultured at 37°C and 30°C.

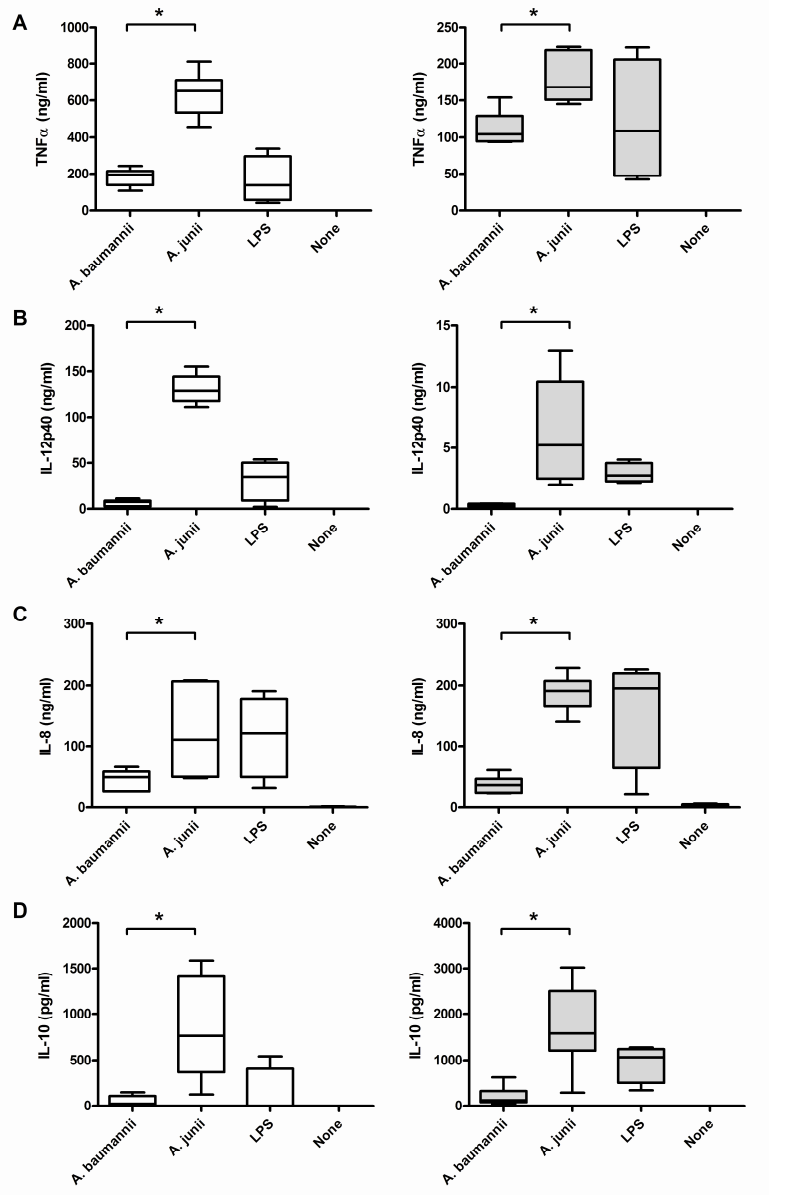

Figure 3. Cytokine production by human macrophages in response to *Acinetobacter*. Boxplots showing TNFα (A), IL-12p40 (B), IL-8 (C) and IL-10 (D) production by cultured human macrophages type 1 (white boxes) and 2 (gray boxes) 24 h after stimulation with strains of *A. baumannii* (n=6), *A. junii* (n=6), LPS and without stimulation (none). Boxes represent medians and second and third interquartiles, whiskers represent range of 6 strains (for *A. baumannii* and *A. junii*) or range of experiments with 4 different donors (for LPS and none). *significant (p≤0.05) difference between *A. baumannii* and *A. junii*.

Discussion

This study was undertaken to obtain some insight into the mechanisms underlying the clinical success of *A. baumannii*. The main conclusion from the present study is that strains of *A. baumannii* induced a poor inflammatory response in human cells, despite the finding that they adhered well to these cells. This conclusion is based on the following findings.

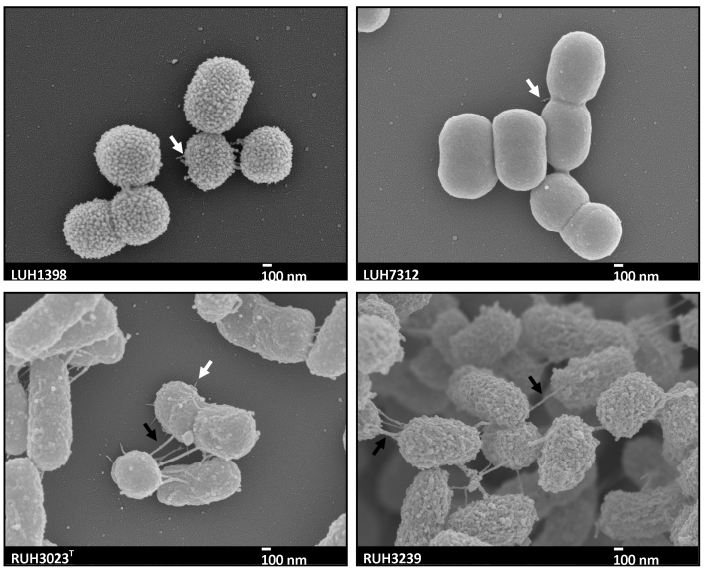

Figure 4. Scanning electron micrographs of *A. baumannii*. Scanning electron micrographs of *A. baumannii* strain LUH1398, LUH7312, RUH3023, and RUH3239. Black arrows indicate long cell extensions; white arrows indicate short pili-like structures. Magnification: 30,000x. Bars: 100 nm.

First, airway epithelial H_{292} cells in vitro produced less IL-6 and IL-8 in response to *A. baumannii* strains than to *A. junii* strains. Furthermore, cultured human macrophages produced less TNFα, IL-12p40, IL-8 and IL-10 in response to *A. baumannii* strains than to *A. junii* strains. In agreement with our in vitro data, Qiu et al documented that the high susceptibility of A/J mice to *A. baumannii* ATCC17961 infection was associated with a reduced local pro-inflammatory response and reduced elimination of bacteria from the lungs [22]. Knapp et al showed in an in vivo model that *A. baumannii* strain RUH2037 induced the release of pro-inflammatory cytokines and chemokines resulting in clearance of bacteria from the lungs of experimentally infected mice [23]. Although study-design and outcome were distinct from our study, the findings emphasize the importance of inflammatory cytokines for clearing of *A. baumannii*. It is furthermore of note that our

finding that clinically relevant strains induced a weak immune response in vitro has also been reported for *Haemophilus influenzae* [24].

There was a wide variation in biofilm formation among a large set of well-described *A. baumannii* strains. Although epidemic strains did not form larger biofilms than sporadic strains, it appeared that strains of European clone II formed larger biofilms than strains of clone I. It is of note that clone II, which was less frequently involved in outbreaks during the 1990s than clone I, is now emerging in several European countries with many strains being carbapenem resistant [25], [26]. Further to *A. baumannii*, the intra-strain variation in biofilm formation of *A.* gen. sp. 3 and 13TU and of the clinically less-relevant species *A. calcoaceticus* and *A. junii* was also considerable. Interestingly, there was no difference between these species, except for *A.* gen. sp. 13TU that formed small biofilms.

Many of the strains used for the current biofilm experiments had previously been used to investigate adherence to H_{292} airway epithelial cells [18]. In that study, it was shown that adherence to airway epithelial cells varied considerably among strains of *A. baumannii*, while strains of clone II had higher adherence values than those of clone I [18]. Likewise, the present study showed a considerable intra-species variation in adherence to human airway epithelial cells but no difference between *A. baumannii* and *A. junii* strains. Biofilm formation on plastic was not correlated to adherence to human cells, indicating that different mechanisms are involved in these colonization processes. Furthermore, the ability to form biofilm on plastic and the capacity to adhere to human cells was not always accompanied by the presence of pilus-like cell surface structures and long cell extensions. Taken together, it seems that biofilm formation and adherence to human cells is strain- and not species-specific. Thus, these features do not solely explain the success of *A. baumannii* in the susceptible host.

In summary, biofilm formation and adherence to airway epithelial cells did not differ between clinically relevant and less-relevant *Acinetobacter* strains and species. However, there was a difference in the production of inflammatory cytokines by airway epithelial cells and macrophages between *A. baumannii* and *A. junii*. This may be a first clue to explain the difference in clinical behavior between *A. baumannii* and *A. junii*. We hypothesize 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.

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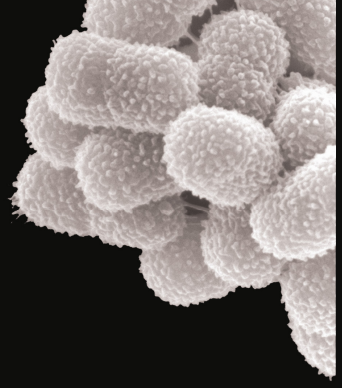

Chapter 3

CsuA/BABCDE-dependent pili are not involved in the adherence of *Acinetobacter baumannii* ATCC19606^T to human airway epithelial cells and their inflammatory response

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Abstract

Acinetobacter baumannii is a nosocomial pathogen responsible for outbreaks of infection worldwide. The factors associated with its ability to colonize/infect human hosts are largely unknown. Adherence to host cells is the first step in colonization/infection, which can be followed by biofilm formation. *A. baumannii* ATCC19606^T biofilm formation on abiotic surfaces depends on expression of the CsuA/BABCDE chaperone–usher pili assembly system. The present study focused on the involvement of CsuA/BABCDE-dependent pili in the interactions between *A. baumannii* 19606^T and human bronchial epithelial cells and sheep erythrocytes. Light microscopy analysis revealed that CsuE-mutant #144 adhered to more bronchial epithelial cells than the parental strain. Similar amounts of interleukin (IL)-6 and IL-8 were produced by bronchial epithelial cells in response to these two bacterial strains. Scanning electron microscopy revealed the presence of two types of surface extensions on ATCC19606^T, i.e., short (29 nm; 5–140 nm) pili and long (260 nm; 143–1008 nm) extensions. The latter were not observed on the CsuE-mutant and therefore are likely the previously described CsuA/BABCDE-encoded extensions. We conclude that CsuA/BABCDE-dependent pili are not involved in adherence of *A. baumannii* ATCC19606^T to bronchial epithelial cells. The structure of the short pili and their possible role in adherence to human cells requires further investigation.

Introduction

During the last decades, *Acinetobacter baumannii* has emerged globally as an important nosocomial pathogen that gives rise to outbreaks of colonization and infection of critically ill, hospitalized patients [3], [6], [12] and [16]. The strains involved are frequently multidrug-resistant (MDR) and have the ability to spread epidemically among patients and survive in the hospital environment [25]. The recent appearance of carbapenem resistance in these strains is a major source of concern [17].

Despite the widely documented problems regarding nosocomial *A. baumannii* infections, little is known about the mechanisms that contribute to the epidemicity and pathogenicity of this species. It is generally thought that the intimate interaction between bacteria and their host begins with the adherence of the microorganism to host tissues followed by colonization of the host [2]. During colonization, bacteria may form microcolonies and produce exopolysaccharides resulting in a highly structured microbial community called biofilm [5]. Bacteria within a biofilm exhibit increased resistance to antimicrobial compounds, including those elicited by the host's immune response [5]. The progression from colonization to infection of the host depends on the balance between the host's immune response and the virulence of the bacterium.

Several in vitro studies have shown that particular strains of *A. baumannii* can adhere to human cells [13] and form biofilms on abiotic surfaces [18], [22], [26] and [27]. Tomaras et al. have demonstrated that the ability of *A. baumannii* strain ATCC19606^T to form pili, 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 [22]. Pili may not only promote adherence and biofilm formation, but the coupling of pili to host cell receptors may also induce inflammation through the production of inflammatory mediators, including chemokines and cytokines [21].

The aim of the present study was to investigate the involvement of *A. baumannii* CsuA/BABCDE-mediated pili in adherence to vertebrate cells and the inflammatory response of human bronchial epithelial cells.

Materials and Methods

Bacterial strains and culture conditions

A. baumannii type strain ATCC19606^T and the *csuE* isogenic insertion mutant ATCC19606 #144 [22] were used. High-resolution fingerprinting by AFLP analysis confirmed that both isolates belong to the same strain (similarity of 99.4% as calculated by the Pearson

correlation coefficient). Upon verification, CsuE-mutant #144 did not form a biofilm on polystyrene after 24 h incubation at 28°C and 37°C. Of note, biofilm formation on abiotic surfaces was tested as described before [22].

Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at −80°C. Prior to each experiment, inocula from frozen cultures were grown overnight at 30°C on sheep blood agar plates (BioMerieux, Boxtel, The Netherlands) or trypticase soy agar (BD, Sparks, MD, USA) containing 5% sheep erythrocytes. For experiments, subcultures were made under these conditions.

Bronchial epithelial cell culture

Human bronchial epithelial H_{292} cells (ATCC CRL-1848, Mansas, VA, USA) were cultured as described previously [13]. Briefly, H_{292} cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 µg/ml streptomycin, 1 mg/ml sodium penicillin G, and 10% heat-inactivated fetal calf serum (FCS) (all from Gibco, Invitrogen, Breda, The Netherlands), further referred to as culture medium, at 37°C/5% CO_2 in 25 cm^2 tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany). At confluency, cells were trypsinized and $2x10^5$ H_{292} cells were resuspended in 0.5 ml of RPMI-1640 supplemented with 2 mM l-glutamine and 10% FCS, further referred to as RPMI medium, in 24-wells plates.

Bacterial adherence to bronchial epithelial cells

The adherence of bacteria to human bronchial epithelial cells was determined as described [13]. Briefly, $2x10^5$ H_{292} cells were cultured for 24 h in RPMI medium on 13-mm diameter plastic coverslips (Thermanox, Nunc, Rochester, NY, USA) placed in 24-well plates. Bacteria were cultured overnight at 30°C on blood agar plates and suspended in RPMI medium to an optical density at 600 nm of 0.6 (SmartSpec 3000, BioRad), corresponding to a concentration of ~$1x10^8$ colony forming units (CFU)/ml, which was verified afterwards by standard vital counting. H_{292} cells were incubated for 1 h at 37°C with $1x10^7$ (range $7x10^6$–$4x10^7$) bacteria. Next, each coverslip was removed and washed five times with prewarmed PBS. The cells on the coverslip were fixed with methanol and stained with Giemsa, after which the coverslips were mounted onto microscope slides. Bacterial adherence was quantitated by light microscopy examination. For each coverslip, a minimum of 800 H_{292} cells were inspected and the percentage of epithelial cells associated with at least one bacterium was determined. In addition, 100 H_{292} cells with attached bacteria were analyzed to assess the number of bacteria associated with human bronchial epithelial cells. Each bacterial strain was examined in duplicate in four independent experiments performed on different days.

Stimulation of bronchial epithelial cells

Cytokine production in bronchial epithelial cells in response to the presence of bacteria was determined using a stimulation assay. Bacteria were cultured overnight at 30°C in Luria–Bertani (LB) medium (10 g bactotryptone, 5 g yeast extract (both from BD, Sparks, MD, USA), 5 g sodium chloride (Merck, Darmstadt, Germany) in 1000 ml distilled water), washed twice with PBS and suspended in RPMI medium to a concentration of $1x10^8$ CFU/ml as calculated from the absorbance of a suspension at 600 nm. Approximately $2x10^5$ H_{292} cells were cultured in 0.5 ml of RPMI medium in 24-well plates. At 85–90% confluency, H_{292} cells were washed once with prewarmed PBS and incubated at 37°C/5% CO_2 with $1x10^7$ bacteria. After 1 h, H_{292} cells were washed five times with prewarmed PBS to remove non-adherent bacteria and incubated again in fresh RPMI medium. After 23 h incubation at 37°C, supernatants were collected and stored at −20°C, until determination of the levels of the inflammatory mediators interleukin (IL)-6 and IL-8 by ELISA. In each experiment, RPMI medium alone was added to the cells in order to obtain background values. Five independent experiments were performed in triplicate.

Determination of inflammatory mediators

The levels of IL-6 and IL-8 in culture supernatants were determined by enzyme-linked immunosorbent assays (ELISA, Biosource, Camarillo, CA, USA) according to the manufacturer's instructions. The lower limit of detection was 15 pg/ml for IL-6 and 7 pg/ml for IL-8.

Scanning electron microscopy

For scanning electron microscopy (SEM), bacteria from an overnight culture on blood agar were suspended in PBS and subsequently fixed for 1 h at room temperature with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1:1). Fixed bacteria were washed twice in PBS and transferred to poly-l-lysine-coated glass slides. After 1 h incubation at room temperature, bacteria were fixed to the slides with 1.5% glutaraldehyde for 30 min at room temperature. Thereafter, slides were washed twice in PBS, dehydrated through a series of graded ethanol, critical-point-dried, and finally coated with a layer of palladium-gold. The bacteria were examined using either a JEOL JSM-6700F or a Zeiss Supra 35VP field emission scanning electron microscope.

Detection of CsuA/B protein

For detection of the CsuA/B protein, bacteria from an overnight culture on blood agar were used to prepare whole-cell lysates as described before [7]. Proteins were size-fractionated on 12.5% polyacrylamide gels [1], transferred to nitrocellulose [24], and

incubated with polyclonal anti-CsuA/B antiserum raised in rabbits using recombinant CsuA/B (overexpressed and purified by Ni-affinity column chromatography) as antigen [23]. The immunocomplexes were detected by chemiluminescence using horse radish peroxidase (HRP)-labeled protein A. Protein concentrations were determined as described previously [4].

Detection of *acuA* gene

The presence of the *acuA* gene, a pilus-encoding gene identified in another *Acinetobacter* species, *A. baylyi*, was determined using a PCR that amplified part of the *acuA* gene (289 bp of 582 bp). Total DNA was extracted from bacteria grown overnight on Iso-Sensitest plates (Oxoid Ltd, Basingstoke, Hampshire, UK) at 30°C. Five bacterial colonies were suspended in 20 µl of lysis buffer (0.25% sodium dodecyl sulfate (SDS), 0.05 N NaOH). The suspension was heated for 15 min at 95°C. After addition of 180 µl of sterile H_2O and centrifugation, supernatants were used for PCR experiments. Amplification reactions were carried out in a total volume of 25 µl containing 5 µl of total DNA, 25 pmol of each primer and 3 U of Taq DNA polymerase (Qiagen, Hilden, Germany). Primers for amplification of the *acuA* gene were $acuA_{forward}$ (5′-CAA CGC TAT GTG CTG CTG G-3′, located at position 32–50 of the *acuA* gene) and $acuA_{reverse}$ (5′-GGC CCA CCC AAA GTA ATC C-3′, located at position 320–302 of the *acuA* gene). Amplification conditions consisted of an initial cycle at 94°C for 1 min followed by 35 cycles of 30 s at 94°C, 40 s at 48°C, 1 min at 72°C, and a final cycle at 72°C for 6 min. PCR products were visualized by electrophoresis and staining with ethidium bromide on 1.5% agarose gels.

Statistical analysis

Results are means ± standard deviations unless indicated otherwise. Data were analyzed for statistical significance using the Wilcoxon rank sum test. p-Values of ≤0.05 were considered significant.

Results

Adhesion to human bronchial epithelial cells and sheep erythrocytes

To investigate whether CsuE-mediated pili are involved in the initial colonization process, adherence of *A. baumannii* 19606^T and its isogenic CsuE-mutant #144 to human bronchial epithelial cells was compared in vitro. Light microscopy analysis of Giemsa-stained cells showed that the CsuE-mutant adhered to a significantly ($p < 0.05$) higher percentage of epithelial cells (29 ± 6.8%) than strain 19606^T (16 ± 3.5%). The number of bacteria per

infected cell was not significantly higher for the mutant (1.9±0.2 bacteria/infected cell) than for the parental strain (1.4±0.1 bacteria/infected cell). Of note, the monolayers and morphology of the bronchial epithelial cells remained intact after 1 h incubation with either of the two *A. baumannii* strains. Differences in inoculum size between experiments did not influence the outcome of the adherence assays (data not shown).

SEM analysis revealed that strain 19606^T and the CsuE-mutant were able to adhere to sheep erythrocytes (Fig. 1), showing that both strains can attach to at least two types of vertebrate cells.

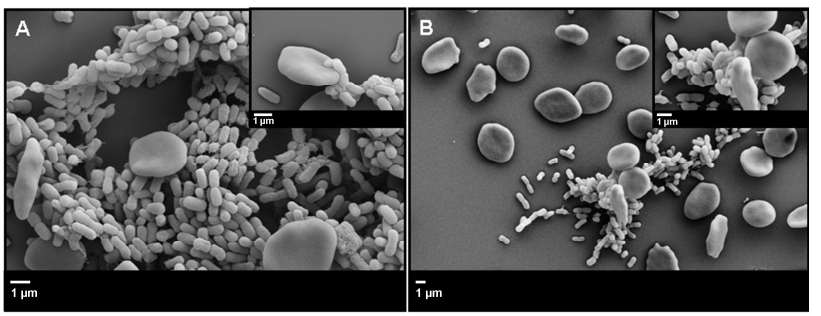

Figure 1. Scanning electron micrographs of *A. baumannii* strain 19606^T (A) and its CsuE-mutant (B) in the presence of sheep erythrocytes. Bars: 1 µm.

Cytokine induction in human bronchial epithelial cells

To investigate whether CsuA/BABCDE-mediated pili contribute to the ability of *A. baumannii* to induce cytokine production by human cells, bronchial epithelial H_{292} cells were exposed to strain 19606^T and its CsuE-mutant in vitro and the levels of IL-6 and IL-8 in the culture supernatant were determined after 24 h. The results revealed that bronchial epithelial cells co-incubated with strain 19606^T or its CsuE-mutant produced significantly ($p<0.05$) higher levels of the cytokine IL-6 (875±398 pg/ml and 823±441 pg/ml, respectively) and the chemokine IL-8 (1751±608 pg/ml and 1930±705 pg/ml, respectively) than unstimulated cells (274±159 pg/ml IL-6 and 428±105 pg/ml IL-8). However, there was no significant difference between the ability of strain 19606^T and the mutant to elicit IL-6 and IL-8 production by bronchial epithelial cells.

Electron microscopy analysis of bacterial surface structures

SEM analysis of bacteria cultured for 16 h at 30°C on blood agar plates revealed the existence of two types of cell appendages in strain 19606^T: thin and short (median 29 nm; range 5–140 nm, n = 20 bacteria) pili-like structures and long cell extensions (Fig. 2A,B).

The latter (median 260 nm, n = 20 bacteria) varied in length from 143 nm to 1008 nm and were irregularly distributed over the cell surface. The CsuE-mutant, which was grown under similar conditions, lacked the long cell extensions and had a more dense distribution of the short pili at its surface than strain 19606^T (Fig. 2D). Of note, short pili could not be observed on all mutant cells. The CsuE-mutant adhered poorly to the poly-l-lysine-coated glass slides used for SEM and bacteria of this strain were mainly found adhering to debris on the glass slide. Interestingly, the short pili were seen mainly at the interface between the bacterial surface and the debris, suggestive of their involvement in adherence. Furthermore, the mutant strain showed, in contrast to the parental strain, only a few cell clusters with no more than two cells grouped together on the surface of the glass slides (Fig. 2C).

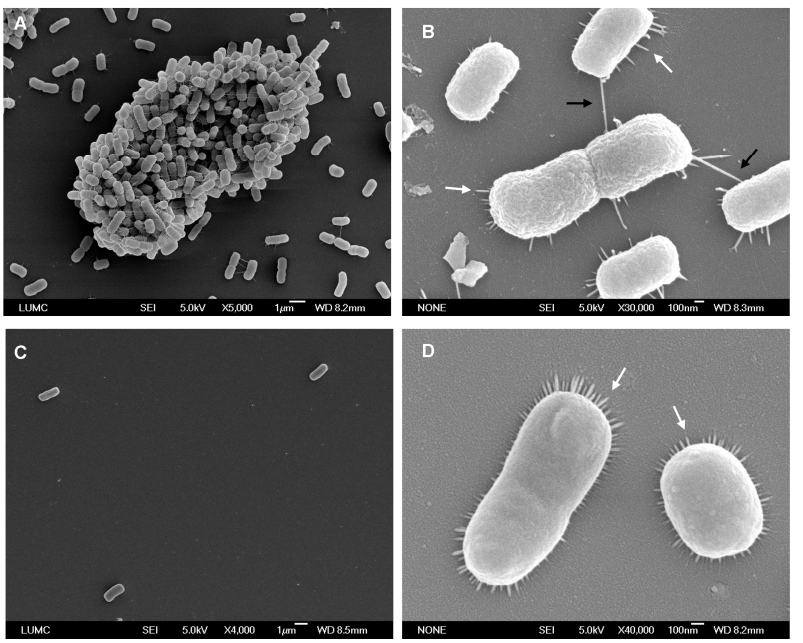

Figure 2. Scanning electron micrographs of *A. baumannii* 19606^T (A and B) and CsuE-mutant (C and D) cells suspended from blood agar plates. Black arrows indicate long cell extensions; white arrows indicate thin and short pili. Bars: 1 µm (A and C) and 100 nm (B and D).

Detection of CsuA/B protein

Since secretion of CsuE, which is the tip adhesion on the pilus, is thought to precede the secretion of the CsuA/B subunit for pilus biogenesis [20], we reasoned that bacteria lacking this secretion event build up the CsuA/B protein in their cytoplasm, leading to

feedback inhibition or degradation of CsuA/B. We assessed the production of CsuA/B by *A. baumannii* 19606^T and its CsuE-mutant by immunoblot analysis of whole lysates with anti-CsuA/B serum. The results demonstrated that the 18-kDa CsuA/B protein is expressed by the parental strain 19606^T. In contrast, the isogenic CsuE-mutant did not express detectable levels of CsuA/B protein (Fig. 3), indicating that the pili produced by the CsuE-mutant are different from those assembled via the CsuA/BABCDE chaperone–usher system.

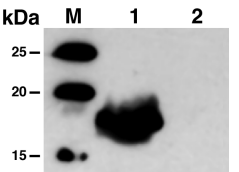

Figure 3. Detection of the CsuA/B protein in *A. baumannii* 19606^T (lane 1) and the CsuE-mutant (lane 2). M, molecular weight markers.

Detection of *acuA* gene

A. baylyi strain ADP1 expresses a chaperone usher system, named Acu, which is required for formation of thin pili in this environmental isolate [8]. To determine whether this system might be involved in formation of CsuA/BABCDE-independent short pili in *A. baumannii*, the presence of *acuA*, which encodes the structural subunit of thin pili in *A. baylyi* [8], was determined using PCR. Although not shown, this approach showed that a 289-bp *acuA* amplicon could be produced when total DNA from *A. baylyi* strain ADP1 was used as a template. In contrast, no amplicon was produced when total DNA from *A. baumannii* strain 19606^T and the CsuE-mutant were used as a template. These observations suggest that the short pili produced by the parental strain 19606^T and the CsuE-mutant are assembled by a system that seems different from CsuA/BABCDE and Acu.

Discussion

In this study, we demonstrated that production of the CsuA/BABCDE-mediated pilus, which is essential for biofilm formation on abiotic surfaces [22], is not required for the adherence of *A. baumannii* ATCC19606^T to H_{292} cells and induction of inflammatory cytokine production by these bronchial epithelial cells. Interestingly, in addition to the CsuA/BABCDE-mediated pili, strain 19606^T was found to produce CsuA/BABCDE-

independent short pili, which to our knowledge have not been reported before in *A. baumannii*.

Our conclusion is based on the following findings. First, light and scanning electron microscopy showed that *A. baumannii* type strain 19606^T and its isogenic CsuE-mutant adhered to human bronchial epithelial cells and sheep erythrocytes in vitro, indicating that CsuA/BABCDE-mediated pili are not essential for adherence to vertebrate cells. The finding that the CsuE-mutant strain adhered to a significantly larger number of epithelial cells than the parental strain might be attributed to increased expression of another type of pilus on the surface of the latter strain, as has been described for *Escherichia coli* [15]. Secondly, we found that *A. baumannii* type strain 19606^T and its CsuE-mutant induced similar levels of inflammatory cytokines IL-6 and IL-8 in bronchial epithelial cells, suggesting that CsuA/BABCDE-mediated pili are not involved in induction of inflammatory responses in human bronchial epithelial cells interacting with this bacterial pathogen.

The presence of pili on the surface of *Acinetobacter* strains was first described by Henrichsen and Blom in 1975 in *A. baylyi* BD4 (formerly *A. calcoaceticus* BD4) [9]. This strain was found to produce bundle-forming thin pili (2–3 nm in diameter) associated with adherence to abiotic and biotic surfaces [8] and individual thick pili (6 nm in diameter) involved in twitching motility [9], i.e., the ability to move on solid surfaces [14] enabling colonization of adjacent surface areas. The involvement of thin pili in adherence to abiotic surfaces was also demonstrated for *A. venetianus* strain RAG-1 [19]. Recently, Ishii et al. described two types of appendages in the highly adhesive *Acinetobacter* sp. strain Tol 5, an anchor-like appendage that tethers the bacterium to the substratum over distances of several hundreds of nanometers, and a peritrichate fibril-type appendage that attaches to the substratum at multiple places fixing the cell at shorter distances [10]. We have extended these findings by describing the existence of two types of cell appendages in the *A. baumannii* type strain 19606^T: (i) irregular long cell extensions connecting bacteria that are assumed to represent the CsuA/BABCDE-mediated pili described by Tomaras et al. [22] and; (ii) thin and short pili-like structures that contact the surface areas adjacent to bacterial cells. The latter pili were not observed by Tomaras et al. [22], possibly because of the use of different experimental conditions and techniques, i.e., Luria–Bertani media and transmission electron microscopy versus blood agar and scanning electron microscopy, which were used to culture bacteria and visualize surface structures, respectively. Interestingly, the short pili were mainly seen at the site where the bacterium adhered to a surface area. Lee et al. [13] also described the presence of thin pili-like extensions on the surface of an *A. baumannii* strain that anchored the bacterium to the membrane surface of human bronchial epithelial cells. These findings raise the challenging question as to

whether the presence of a particular surface area triggers the production of short pili by *A. baumannii*, as has been described for *Acinetobacter* sp. strain Tol 5 [11].

It should be noted that although no PCR product could be amplified, *acuA*, which encodes pili in *A. baylyi* that are related to F17 pili of *Escherichia coli* [8], still could be present in *A. baumannii* 19606^T. This is supported by the finding that a genome search of the *A. baumannii* AB307-0294 genome using *acuA* from *A. baylyi* leads to detection of genes encoding subunits of F17-related pili.

In summary, in addition to the already described CsuA/BABCDE-mediated pilus that plays a role in biofilm formation on abiotic surfaces, *A. baumannii* ATCC19606^T produces a CsuA/BABCDE-independent thin and short pilus, which may be involved in adherence of the bacterium to biotic surfaces such as those of human respiratory cells. Further genetic, structural and physiological studies are necessary to elucidate the role of this thin pilus in the pathobiology of *A. baumannii*.

Acknowledgments

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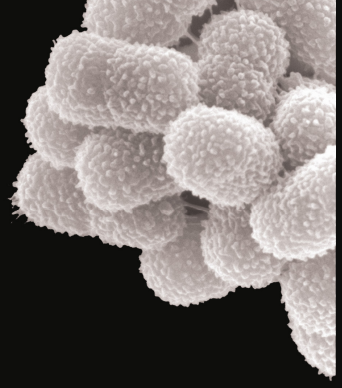

Chapter 4

Differences in *Acinetobacter baumannii* strains and host innate immune response determine morbidity and mortality in experimental pneumonia

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Abstract

Despite many reports documenting its epidemicity, little is known on the interaction of *Acinetobacter baumannii* with its host. To deepen our insight into this relationship, we studied persistence of and host response to different *A. baumannii* strains including representatives of the European (EU) clones I-III in a mouse pneumonia model. Neutropenic mice were inoculated intratracheally with five *A. baumannii* strains and an *A. junii* strain and at several days morbidity, mortality, bacterial counts, airway inflammation, and chemo- and cytokine production in lungs and blood were determined. *A. baumannii* RUH875 and RUH134 (EU clone I and II, respectively) and sporadic strain LUH8326 resulted in high morbidity/mortality, whereas *A. baumannii* LUH5875 (EU clone III, which is less widespread than clone I and II) caused less symptoms. *A. baumannii* type strain RUH3023^T and *A. junii* LUH5851 did not cause disease. All strains, except *A. baumannii* RUH3023^T and *A. junii* LUH5851, survived and multiplied in the lungs for several days. Morbidity and mortality were associated with the severity of lung pathology and a specific immune response characterized by low levels of anti-inflammatory (IL-10) and specific pro-inflammatory (IL-12p40 and IL-23) cytokines at the first day of infection. Altogether, a striking difference in behavior among the *A. baumannii* strains was observed with the clone I and II strains being most virulent, whereas the *A. baumannii* type strain, which is frequently used in virulence studies appeared harmless.

Introduction

Multidrug-resistant *Acinetobacter baumannii* is a cause of severe infections in critically ill patients and notorious for its ability to spread epidemically. Three clonal lineages of *A. baumannii*, European (EU) clone I, II and III, are implicated in outbreaks worldwide [1,2]. Other *Acinetobacter* species, including the skin colonizer *A. junii* [3], are only incidentally involved in infection [4].

Various factors are assumed to contribute to the ability of *A. baumannii* to colonize the hospital environment and patients [5–11]. However, knowledge on the host's response to *A. baumannii* is limited. Recognition by Toll-like receptor 4 and CD14 [12] and early recruitment of neutrophils [13,14] are important factors in the host innate defence against respiratory *A. baumannii* infection in mice. Others demonstrated the differential ability of clinical *A. baumannii* isolates to induce severe infections in neutropenic mice [15]. We previously showed in vitro that *A. baumannii* strains induce significantly less inflammatory cytokine production in human airway epithelial cells and cultured human macrophages than *A. junii* strains do [5], emphasizing the role of the innate immune system in *A. baumannii* infections.

The aim of the present study was to investigate the virulence of and host innate immune response to well-characterized *A. baumannii* strains, including representatives of clones I-III, and an *A. junii* strain in a mouse pneumonia model.

Materials and Methods

Bacteria

Five *A. baumannii* strains, including reference strains of EU clones I-III, the type strain (RUH3023^T=ATCC19606^T) and a sporadic isolate (LUH8326), and one *A. junii* strain were investigated (Table 1). Bacteria were preserved in glycerolbroth at -80°C. Prior to experiments, strains were rendered virulent by a single passage in mice.

Animals

Specific pathogen-free female C3H/HeN mice weighing 18-20 g were housed fifteen per cage and had *ad libitum* access to chow and water throughout the experiments. Animal studies were approved by the Animal Experimental Committee of the Angers University Hospital (permit C49007002) and complied with relevant laws related to the conduct of animal experiments.

Table 1. Strain characteristics

Strain	City (country)		Year	Specimen	EU Clone*	Outbreak†	MDR‡
A. baumannii							
RUH875	Dordrecht	(NL)	1984	urine	I	+	+
RUH134	Rotterdam	(NL)	1982	urine	II	+	+
LUH5875	Utrecht	(NL)	1997	blood	III	+	+
LUH8326	Leiden	(NL)	2002	wound	-	-	-
RUH3023T (ATCC19606T)	Atlanta	(USA)	1965	urine	-	-	-
A. junii							
LUH5851	Leiden	(NL)	1999	ear	-	-	-

*Strain belonging to European clones I-III (+) or not belonging to these clones (-). All isolates have been identified to species by one or more genotypic methods [1,33,34]. †Outbreak-associated (+) strain (i.e., common AFLP profile in >2 patients and with same time-space-origin) or (-) sporadic strain. ‡Multidrug-resistant (+) strain (i.e., resistant to more than two of the following drug classes or combinations: cephalosporins, carbapenems, ampicillin-sulbactam, quinolones and aminoglycosides) or (-) susceptible strain.

Mouse pneumonia

The survival of *Acinetobacter* strains after intratracheal infection of mice was assessed according to Eveillard et al [15]. To favour the onset of infection, mice were rendered transiently neutropenic by intraperitoneal injection with 150 mg of cyclophosphamide per kg of body weight (in 100 µl of saline) at days 4 and 3 prior to infection. Bacteria from an overnight culture on blood agar were suspended into saline to an optical density of 0.5 McFarland, corresponding to a concentration of approximately 10^8 colony forming units (CFU)/ml. Mice were anesthesized by isoflurane in conjunction with oxygen and 50 µl of the bacterial suspension were injected intratracheally via a cannula. Immediately after inoculation, two animals were sacrificed, lungs were homogenized and vital count was performed to verify the infection inoculum (range $5.9x10^5$ - $4.7x10^6$ CFU/g lung tissue). At 1, 2, 3 and 4 days after infection, if possible 8 mice per strain were anesthesized and blood was collected by intracardiac puncture, after which mice were sacrificed by cervical dislocation. Serum was obtained by centrifugation of blood samples and stored at -80°C for cytokine analysis. Spleens and part of the lungs were removed, weighed and homogenized in 3 ml of phosphate buffered saline (pH 7.4) using the GentleMACS Dissociator (Miltenyi Biotec, Germany). Vital counts in blood, lung and spleen homogenates were performed to assess the number of viable bacteria (lowest limit of detection: 20 CFU/ml). Lung homogenates were stored at -80°C for cytokine analysis.

A semi-quantitative analysis of mice morbidity was performed using a clinical score ranging from 0 for no clinical symptoms to 4 for maximal symptoms based on the following criteria: mice mobility (0, spontaneous; 1, only after stimulation; 2, absent), the development of conjunctivitis (0, absent; 1, present), and the aspect of the hair (0,

normal; 1 ruffled). Mortality was assessed daily and analyzed by Kaplan-Meier survival curve.

Histological analysis of lung inflammation

Lungs of mice at day 1-4 after inoculation were analyzed by histological examination as described [15]. A semi-quantitative analysis of the lung tissue damage was performed by grading five random 20x fields of hematoxylin/eosin-stained sections according to the following criteria: alveolar wall destruction [absent (0); <25% (1), 25-75% (2), >75% (3) of alveoli destructed], infiltration by leukocytes [absent (0); <20 (1), 20-50 (2), >50 (3) per field] and hemorrhage [absent (0); mild (1); moderate (2); severe(3)]. The sum of scores represents the lung pathology score ranging from 0 for no pathology to 9 for severe pathology.

Determination of inflammatory mediators

Levels of interleukin (IL)-1β, IL-6, IL-10, IL-12p40, IL-13, IL-23, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-1α, MIP-2, regulated upon activation, normal T cell expressed and secreted (RANTES) and tumor necrosis factor (TNF)α in serum and lung homogenates were determined using multiplexing xMAP technology (Luminex Corporation Austin, USA). Multiplex kits were from Millipore (Millipore Corporation, USA).

Statistical analysis

Data were analyzed using the Kruskal-Wallis one-way analysis of variance and Wilcoxon rank sum test (SPSS 17.0). Mortality data were analyzed by Cox-regression. Spearman rank correlation coefficients were calculated to evaluate associations between parameters. $P \leq 0.05$ were considered significant.

Results

Morbidity and mortality

Mice infected with the different *A. baumannii* strains and the *A. junii* strain (Table 1) were monitored daily for morbidity and mortality. Mice infected with *A. baumannii* RUH3023^T or *A. junii* LUH5851 showed virtually no signs of morbidity (Table 2). In contrast, infection with *A. baumannii* clone I (RUH875) and clone II (RUH134) caused high morbidity already at the first day of infection, which remained high during the second day.

Table 2. Morbidity and lung pathology associated with *Acinetobacter* respiratory infection

	A. baumannii					*A. junii*
	RUH875 (clone I)	RUH134 (clone II)	LUH5875 (clone III)	LUH8326 (sporadic)	RUH3023T	LUH5851
Clinical score*						
Day 1	2.7 ± 1.0	2.9 ± 0.9	1.9 ± 1.2	2.3 ± 0.9	0.0 ± 0.2	0.0 ± 0.2
Day 2	3.2 ± 0.6	2.7 ± 1.2	2.2 ± 1.2	3.3 ± 0.5	0	0.1 ± 0.2
Day 3	NA	NA	1.9 ± 1.3	1.5 ± 1.7	0	0
Day 4	NA	NA	0.2 ± 0.4	NA	0	0
Lung pathology score†						
Day 1	8.2 ± 0.7	8.0 ± 0.9	6.8 ± 0.5	7.2 ± 0.0	2.1 ± 0.9	3.6 ± 1.1
Day 2	8.0 ± 0.9	8.3 ± 0.8	6.9 ± 1.5	7.1 ± 0.3	2.1 ± 0.6	3.0 ± 1.2
Day 3	NA	NA	7.3 ± 0.6	7.5 ± 1.2	2.2 ± 0.2	2.7 ± 0.6
Day 4	NA	NA	6.7 ± 1.0	NA	2.4 ± 0.4	2.5 ± 0.5

*Morbidity was recorded daily using a clinical score, which includes mobility, the development of conjunctivitis, and aspects of the hair and ranges from 0 for no clinical symptoms to 4 for maximal symptoms. †Sections of lungs of mice were stained with haematoxylin & eosin and lung tissue damage was assessed using the lung pathology score, which includes alveolar wall destruction, leukocyte infiltration and hemorrhage and ranges from 0 for no pathology to 9 for severe pathology. Values are means ± standard deviations for 8 mice except for LUH8326 at day 3, where n=4. Values are representative for surviving mice only. NA, not assessable, due to the high mortality associated with these strains.

Infection with clone III (LUH5875) and sporadic isolate LUH8326 was accompanied by significantly ($p<0.01$) less morbidity at the first day of infection. For LUH8326, morbidity increased during the second day of infection but for LUH5875 it remained low (Table 2).

Mice infected with RUH3023^T or *A. junii* LUH5851 did not die, whereas mortality was very high among mice infected with LUH8326, RUH875, and RUH134 (52%, 72% and 86%, respectively, Fig. 1). Less animals (28%) died after infection with LUH5875 ($p<0.05$, Fig. 1). Most animals died within the first 2 days of infection with RUH875, RUH134 and LUH8326 and between days 3-4 of infection with LUH5875. Of note, the results described in the next paragraphs are representative for the surviving mice only.

Persistence of *Acinetobacter* strains in lungs and extrapulmonary dissemination

After 24 h of infection, RUH875, RUH134, LUH5875 and LUH8326 had multiplied in the lungs to approximately 1x10^9 CFU/g of lung (Fig. 2A). These strains persisted in the lungs up to day 3-4 of infection (Fig. 2A). In contrast, RUH3023^T was cleared from the lungs already within the first day of infection. The levels of *A. junii* LUH5851 remained stable during the first day of infection, declined sharply thereafter with complete clearance by day 3.

RUH875, RUH134, LUH5875, LUH8326 and *A. junii* LUH5851 were found in blood and spleen after the first day of infection (Fig. 2B, C). The bacterial load in the spleen after the first day of infection was significantly ($p<0.05$) higher for RUH875 than for LUH5875.

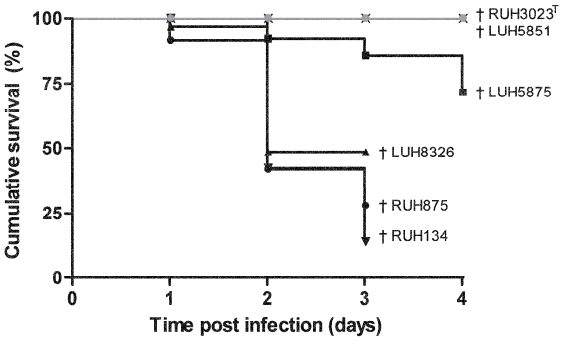

Figure 1. Survival associated with *Acinetobacter* respiratory infection. Survival of mice after intratracheal infection with *A. baumannii* clone I strain RUH875, clone II strain RUH134, clone III strain LUH5875, sporadic strain LUH8326 and type strain RUH3023^T, and *A. junii* strain LUH5851. Results are expressed as percentage survival at days 1-4 of infection. †, all mice dead/sacrificed.

A. junii LUH5851 disseminated into the blood and spleen to a significantly ($p<0.05$) lower extent than RUH875, RUH134, LUH5875 and LUH8326. For these *A. baumannii* strains, levels in blood and spleen remained stable up to day 3-4, whereas *A. junii* LUH5851 was cleared completely from blood and spleen already at day 2 of infection. *A. baumannii* RUH3023^T did not disseminate into the blood and spleen.

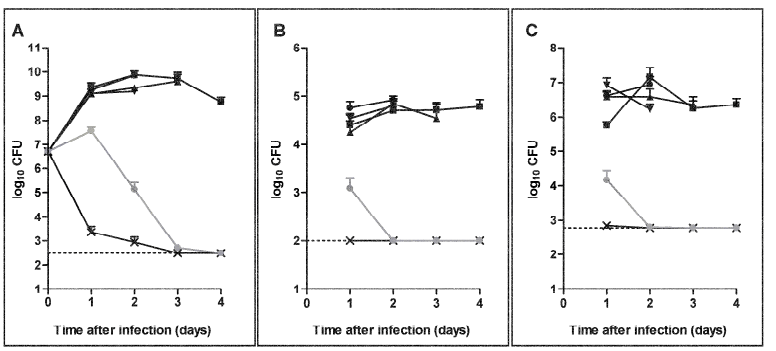

Figure 2. Persistence of *Acinetobacter* strains in the lungs and extrapulmonary dissemination. Levels of *A. baumannii* clone I strain RUH875 (black circles), clone II strain RUH134 (black downward triangles), clone III strain LUH5875 (black squares), sporadic strain LUH8326 (black upward triangles) and type strain RUH3023^T (black crosses), and *A. junii* strain LUH5851 (gray circles) in the lungs (A), bloodstream (B) and spleen (C) of mice at 1 to 4 days after intratracheal injection. Results are expressed as mean CFU per g of tissue (for lung and spleen) or CFU per ml (for blood) ± standard errors of the mean of 8 mice except for LUH8326 at 3 days after infection, where n=4. Values are representative for surviving mice only. Dotted lines represent the lowest limit of detection.

Overall, bacterial counts in lungs correlated ($p<0.01$) to those in the bloodstream ($r=0.85$) and spleen ($r=0.85$). Furthermore, morbidity and mortality were associated ($p<0.01$) with bacterial counts in the lungs ($r=0.77$ and $r=0.62$, respectively), bloodstream ($r=0.86$ and $r=0.75$, respectively) and spleen ($r=0.85$ and $r=0.82$, respectively).

Lung pathology

Histologic examination of the lungs of mice at the first day of infection with RUH875, RUH134, LUH5875 and LUH8326 revealed hypercellularity due to increased numbers of lymphocytes, monocytes and macrophages, and thickened alveolar walls (Fig. 3, Table 2). The lungs of mice infected with these strains were highly consolidated and many areas had hemorrhagic lesions. The severity of tissue damage remained stable over time for all strains (Table 2). Alveolar wall destruction was more severe after the first day of infection with RUH875 and RUH134 than with LUH5875 and after the second day of infection with RUH875 and RUH134 than with LUH8326. Infection with RUH3023^T and *A. junii* LUH5851 resulted in significantly ($p<0.05$) less lung damage (Table 2).

Overall, severity of tissue damage was correlated ($p<0.001$) to bacterial levels in the lungs ($r=0.81$), morbidity ($r=0.81$) and mortality ($r=0.84$).

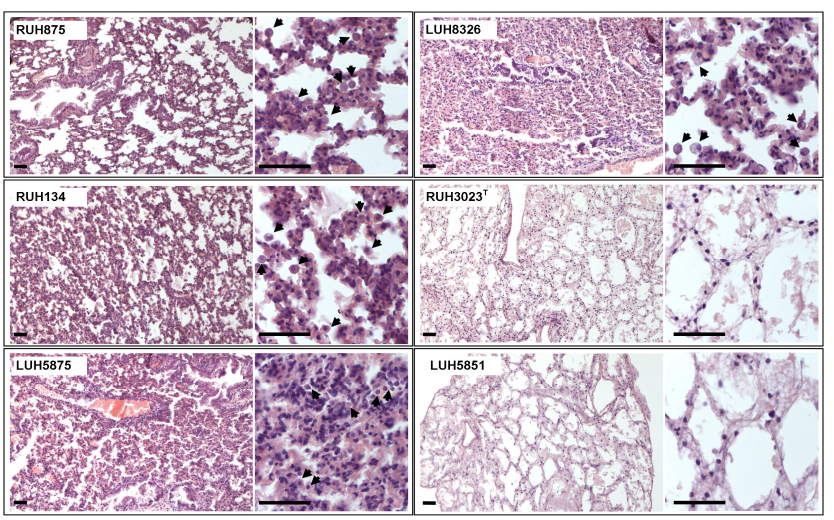

Figure 3. Light micrographs of lungs of mice infected with *Acinetobacter*. Sections of the lungs of mice at 1 day after infection with *A. baumannii* clone I strain RUH875, clone II strains RUH134, clone III strain LUH5875, sporadic strain LUH8326, type strain RUH3023^T or *A. junii* strain LUH5851, stained with haematoxylin-eosin. Arrows indicate inflammatory cell infiltrates. Bars, 50 µm.

Inflammatory response to *Acinetobacter*

After the first day of infection with *A. baumannii* strains RUH875, RUH134, LUH5875 and LUH8326, levels of all chemo- and cytokines in the lungs were substantially elevated ($p<0.05$) as compared to basal levels. The levels of the chemokines KC, MIP-1α, MIP-2, and the pro-inflammatory cytokines IL-1β, IL-6 and TNFα further increased and peaked at day 2 of infection (Table 3).

Table 3. Inflammatory response in lungs of mice infected with four virulent *A. baumannii* strains

		RUH875 (clone I)		RUH134 (clone II)		LUH5875 (clone III)		LUH8326 (sporadic)	
Chemokines	**Day**								
KC†	**1**	386	(38 - 700) *	311	(198 - 474) *	377	(4 - 555) *	310	(28 - 493) *
basal: 17 (3 - 57)	**2**	326	(150 - 749) *	236	(16 - 306) *	186	(25 - 540) *	204	(7 - 1753) *
	3	NA		NA		52	(8 - 224)	45	(32 - 422)
	4	NA		NA		41	(4 - 45)	NA	
MIP-1α†	**1**	142	(1 - 404)	114	(13 - 287) *	112	(13 - 217) *	147	(6 - 201) *
basal: 2 (1 - 5)	**2**	589	(118 - 888) *	319	(5 - 1076) *	760	(3 - 909) *	196	(2 - 765) *
	3	NA		NA		78	(12 - 654) *	124	(90 - 596) *
	4	NA		NA		114	(5 - 191) *	NA	
MIP-2†	**1**	171	(4 - 246) *	145	(71 - 372) *	119	(3 - 257) *	137	(6 - 220) *
basal: 5 (1 - 45)	**2**	400	(200 - 755) *	342	(4 - 591) *	365	(6 - 546) *	312	(3 - 406) *
	3	NA		NA		79	(4 - 253) *	109	(86 - 219) *
	4	NA		NA		97	(3 - 202) *	NA	
RANTES††	**1**	1753	(975 - 5282) *	1682	(893 - 2708) *	2124	(1406 - 2452) *	1885	(996 - 3340) *
basal: 236 (221 - 501)	**2**	1229	(794 - 1760) *	1278	(671 - 1773) *	1559	(1475 - 1627) *	1000	(812 - 1381) *
	3	NA		NA		930	(691 - 1010) *	958	(665 - 2027) *
	4	NA		NA		834	(646 - 850) *	NA	
Pro-inflammatory cytokines									
IL-1β†	**1**	20	(5 - 60) *	19	(7 - 66) *	18	(5 - 31) *	30	(5 - 48) *
basal: 1 (1 - 2)	**2**	51	(13 - 264) *	33	(3 - 135) *	69	(6 - 268) *	24	(2 - 81) *
	3	NA		NA		19	(3 - 84) *	28	(12 - 75) *
	4	NA		NA		14	(4 - 20) *	NA	
IL-6†	**1**	133	(3 - 217) *	67	(19 - 280) *	85	(2 - 181) *	119	(1 - 174) *
basal: 3 (2 - 11)	**2**	127	(43 - 670) *	76	(4 - 395) *	98	(0.4 - 376) *	72	(1 - 151) *
	3	NA		NA		10	(1 - 217)	6	(3 - 266)
	4	NA		NA		9	(1 - 14)	NA	
IL-12p40††	**1**	1901	(1202 - 3091) *	2249	(743 - 4130) *	2824	(1818 - 5251) *	3698	(1900 - 5365) *
basal: 203 (163 - 237)	**2**	1730	(377 - 2380) *	1513	(976 - 2644) *	1991	(1093 - 3005) *	1031	(834 - 1282) *
	3	NA		NA		669	(381 - 1853) *	760	(415 - 2210) *
	4	NA		NA		319	(116 - 494)	NA	
IL-23†	**1**	99	(58 - 113) *	82	(48 - 122) *	150	(104 - 175) *	113	(98 - 150) *
basal: 11 (6 - 17)	**2**	73	(33 - 227) *	86	(59 - 108) *	79	(46 - 112) *	63	(36 - 89) *
	3	NA		NA		43	(19 - 135) *	59	(23 - 169) *
	4	NA		NA		26	(6 - 61)	NA	
TNFα†	**1**	10	(0.1 - 17)	8	(1 - 22) *	11	(1 - 30) *	12	(0.4 - 17) *
basal: 0.4 (0.2 - 5)	**2**	15	(3 - 28) *	11	(0.4 - 32) *	15	(1 - 32) *	6	(0.4 - 21) *
	3	NA		NA		2	(0.3 - 35)	2	(2 - 23)
	4	NA		NA		4	(1 - 9)	NA	
Anti-inflammatory cytokines									
IL-10††	**1**	824	(416 - 2126) *	991	(578 - 1724) *	2445	(1834 - 4053) *	2168	(1348 - 3753) *
basal: 273 (157 - 355)	**2**	792	(389 - 4168) *	1346	(568 - 1923) *	2016	(844 - 2741) *	876	(541 - 1837) *
	3	NA		NA		2923	(862 - 1234) *	1903	(862 - 3330) *
	4	NA		NA		3740	(1154 - 4051) *	NA	
IL-13††	**1**	976	(678 - 2073) *	1481	(567 - 3274) *	2739	(571 - 3691) *	1628	(531 - 3958) *
basal: 215 (173 - 251)	**2**	1456	(565 - 4134) *	792	(275 - 1145) *	1368	(282 - 1765) *	527	(244 - 959) *
	3	NA		NA		262	(207 - 490)	327	(194 - 592)
	4	NA		NA		338	(123 - 511)	NA	

Mice were intratracheally infected with *A. baumannii* RUH875, RUH134, LUH5875 or LUH8326 for 1-4 days. Levels of inflammatory mediators were determined in the lung homogenates of mice directly after instillation (basal values), and 1-4 days after instillation. Results are medians and ranges for 8 mice, except for LUH8326 at 3 days after infection, where n=4. Values are representative for surviving mice only. NA, not assessable, due to the high mortality associated with these strains. *, significantly ($p<0.05$) different from basal level. **†**, results in ng/g of lung tissue; **††**, results in pg/g of lung tissue.

The kinetics of production of the anti-inflammatory IL-10 differed significantly in animals infected with the four strains with a peak at day 1 in animals infected with LUH8326, at day 2 after infection with RUH134 and at day 3 after infection with LUH5875. At the first day of infection, levels of IL-10 in lungs were significantly ($p<0.05$) lower in mice infected with RUH875 and RUH134 as compared to infection with LUH5875 and LUH8326. IL-10 levels remained lower after the second day of infection with RUH875 and RUH134 as compared to LUH5875. Moreover, levels of the anti-inflammatory IL-13 in lungs of mice infected for 1 day with RUH875 tended to be lower ($p=0.1$) than with LUH5875. In addition to these anti-inflammatory cytokines, lower ($p<0.05$) levels of the pro-inflammatory IL-12p40 and IL-23 were seen in lungs of mice infected for 1 day with RUH875 and RUH134 than with LUH5875 and LUH8326. *A. baumannii* RUH3023^T and *A. junii* LUH5851 did not induce cyto- and chemokine production (data not shown).

In serum, all cyto- and chemokine levels were elevated 1 day after infection with RUH875, RUH134, LUH5875 and LUH8326, except for IL-23 that was not detectable (Table S1 in the online data supplement). For the majority of the cyto- and chemokines measured (KC, MIP-1α, MIP-2, IL-1β, IL-6 and TNFα), levels in the lungs corresponded to levels in serum. However, the kinetics of the innate response in lungs and serum differed, with some cyto- and chemokines peaking earlier (IL-1β and IL-10) and some later (MIP-1α, MIP-2, RANTES and TNFα) in serum than in the lungs of infected animals. No significant differences in levels of inflammatory mediators were seen between RUH875, RUH134, LUH5875 and LUH8326. Infection of mice with RUH3023^T and *A. junii* LUH5851 did not cause an increase in inflammatory mediators in serum, except for a 5-fold increase of RANTES and IL-1β levels (Table S1).

Overall, cyto- and chemokine levels in lungs and serum correlated ($p<0.05$) to bacterial levels in lungs and blood, respectively. We determined whether there was a correlation between cyto- and chemokine production at day 1 and tissue pathology, morbidity and mortality of mice at days 1-3 after infection with *A. baumannii* RUH875, RUH134, LUH5875, and LUH8326. Results revealed that low levels of IL-10 in lungs of mice were associated with severe lung pathology ($r=-0.70$, $p<0.05$) at the first day of infection. Morbidity was associated with low IL-10 levels ($r=-0.72$, $p<0.001$), low IL-12p40 (-0.37, $p<0.05$) and IL-23 ($r=-0.67$, $p<0.001$) levels in the lungs. Moreover, low levels of IL-10 in lungs at day 1 correlated to mortality at days 2 ($r=-0.77$; $p<0.05$) and 3 of infection ($r=-0.72$, $p<0.01$). A similar correlation was found between IL-12p40 and IL-23 levels at day 1 and mortality at days 2 ($r=-0.37$, $p<0.05$ and $r=-0.71$, $p<0.01$, respectively) and 3 ($r=-0.35$, $p<0.05$ and $r=-0.67$, $p<0.01$, respectively).

Table S1. Inflammatory response in serum of mice infected with *Acinetobacter*

		RUH875		RUH134		LUH5875		LUH8326		RUH3023T		LUH5851	
Chemokines	**Day**												
KC	**1**	60	(7 - 256) *	57	(7 - 251) *	46	(1 - 346) *	67	(7 - 145) *	1	(0.2 - 4)	8	(0.2 - 11) *
basal: 0.1 (0.03 - 0.6)	**2**	21	(4 - 54) *	9	(2 - 674) *	12	(2 - 80) *	6	(0.2 - 72) *	0.2	(0.1 - 0.4)	0.4	(0.2 - 5)
	3	NA		NA		0.1	(0 - 93)	0.01	(0 - 0.02)	0	(0 - 0.03)	0	(0 - 0.2)
	4	NA		NA		0.1	(0 - 1)	NA		0		0	
MIP-1a	**1**	0.4	(0.1 - 2) *	0.3	(0.1 - 1) *	0.2	(0.1 - 0.3) *	0.1	(0.1 - 0.3) *	0.1		0.1	
basal: 0.1	**2**	2	(1 - 11) *	2	(0.1 - 5) *	4	(0.1 - 17) *	1	(0.1 - 4) *	0.1		0.1	
	3	NA		NA		7	(0.1 - 84) *	2	(0.4 - 82) *	0.1		0.1	
	4	NA		NA		6	(0.1 - 23) *	NA		0.1		0.1	
MIP-2	**1**	18	(1 - 25) *	11	(2 - 23) *	5	(0.1 - 13) *	11	(2 - 19) *	0.2	(0.1 - 0.2)	0.2	(0.1 - 1)
basal: 0.1 (0.1 - 0.2)	**2**	10	(1 - 22) *	8	(0.1 - 18) *	10	(0.3 - 14) *	3	(0.1 - 10)	0.1		0.1	
	3	NA		NA		0.5	(0.1 - 65)	0.1	(0.1 - 58)	0.1		0.1	(0.1 - 0.2)
	4	NA		NA		0.1	(0.1 - 1)	NA		0.1		0.1	
RANTES	**1**	49	(4 - 127) *	31	(12 - 101) *	19	(4 - 53) *	23	(4 - 50) *	18	(12 - 32) *	25	(9 - 46) *
basal: 4 (4 - 6)	**2**	27	(4 - 59)	21	(4 - 126) *	22	(4 - 50) *	16	(4 - 38)	13	(12 - 21) *	15	(12 - 19)
	3	NA		NA		10	(4 - 113)	6	(4 - 110)	9	(4 - 21)	8	(4 - 32) *
	4	NA		NA		15	(12 - 30) *	NA		11	(9 - 18) *	12	(9 - 21) *
Pro-inflammatory cytokines													
IL-1b	**1**	393	(5 - 845) *	185	(102 - 550) *	234	(5 - 617) *	225	(55 - 1002) *	119	(119 - 223) *	62	(5 - 119)
basal: 30 (5 - 55)	**2**	144	(55 - 617) *	144	(5 - 584)	411	(55 - 684) *	78	(55 - 908) *	62	(5 - 119)	119	(119 - 223) *
	3	NA		NA		78	(5 - 1002)	30	(5 - 1495)	119	(5 - 119)	119	(5 - 119) *
	4	NA		NA		119	(56 - 223) *	NA		119	(5 - 223)	119	(5 - 223) *
IL-6	**1**	33	(2 - 99) *	27	(7 - 95) *	30	(1 - 84) *	40	(1 - 105) *	0	(0 - 1)	5	(0 - 10)
basal: 0.01 (0.01 - 0.1)	**2**	27	(9 - 79) *	16	(0.3 - 633) *	34	(5 - 181) *	8	(0 - 158) *	0		0	(0 - 1)
	3	NA		NA		1	(0 - 151) *	1	(0.1 - 134) *	0		0	(0 - 0.1)
	4	NA		NA		0.2	(0 - 4)	NA		0		0	
IL-12p40	**1**	42	(20 - 173) *	27	(20 - 85)	25	(20 - 75)	20	(20 - 28)	20		20	(20 - 22)
basal: 20	**2**	20	(20 - 61)	20	(20 - 72)	20	(20 - 50)	20	(20 - 57)	20		20	
	3	NA		NA		20	(20 - 85)	20		20		20	
	4	NA		NA		20	(20 - 27)	NA		20		20	
TNFa	**1**	128	(3 - 285) *	84	(5 - 223) *	70	(3 - 97) *	55	(3 - 110) *	3		24	(3 - 24) *
basal: 3	**2**	259	(84 - 345) *	191	(3 - 446) *	185	(3 - 450) *	77	(3 - 540) *	3	(3 - 24)	3	(3 - 24)
	3	NA		NA		121	(3 - 3749) *	21	(3 - 2295)	3	(3 - 24)	3	(3 - 24)
	4	NA		NA		24	(3 - 234) *	NA		3	(3 - 82)	3	(3 - 24)
Anti-inflammatory cytokines													
IL-10	**1**	992	(45 - 1271) *	373	(16 - 2127) *	45	(16 - 1023) *	159	(45 - 528) *	20	(16 - 124) *	20	(20 - 92) *
basal: 16	**2**	620	(237 - 1299) *	147	(35 - 519) *	319	(16 - 1528) *	159	(16 - 552) *	18	(16 - 20)	20	(20 - 75) *
	3	NA		NA		390	(16 - 45053) *	305	(16 - 1541) *	20	*	20	(16 - 58)
	4	NA		NA		1475	(20 - 3020) *	NA		30	(20 - 92) *	20	(16 - 58) *
IL-13	**1**	2	(1 - 3) *	2	(1 - 2)	2	(1 - 2)	2	(1 - 3) *	2	(1 - 2)	1	(1 - 2)
basal: 1 (1 - 2)	**2**	1	(1 - 2)	2	(1 - 2)	2	(1 - 4)	2	(1 - 2) *	2	(1 - 2)	1	(1 - 2)
	3	NA		NA		1	(1 - 2)	1	(1 - 2)	1	(1 - 2)	1	(1 - 2)
	4	NA		NA		1	(1 - 2)	NA		1	(1 - 2)	1	(1 - 2)

Mice were intratracheally infected with *A. baumannii* RUH875, RUH134, LUH5875, LUH8326, RUH3023^T or *A. junii* LUH5851 for 1-4 days. Levels of inflammatory mediators were determined in the serum of mice directly after instillation (basal values), and 1-4 days after instillation. Results are median and ranges for 8 mice, except for LUH8326 at 3 days after infection, where n=4. Values are representative for surviving mice only. NA, not assessable, due to the high mortality associated with these strains. *, significantly ($p < 0.05$) different from basal level.

Discussion

The outcome of pneumonia differed strikingly among *A. baumannii* strains with clone I and II and the sporadic isolate being highly virulent and the clone III strain (a clone less widespread than clones I and II) being less virulent. Infection of mice with type strain RUH3023^T and *A. junii* LUH5851 did not result in morbidity/mortality.

The clone I, II, III strains and the sporadic strain survived and multiplied in the lungs and disseminated to the bloodstream at high levels. Difference in mortality between clone III versus clone I, II and the sporadic strain could not be attributed to bacterial loads in lungs or blood, suggesting that proliferation in lungs and extrapulmonary dissemination are not the only factors contributing to the virulence of these strains.

We previously demonstrated in vitro that *A. junii* strains induced a stronger innate immune response in human cells than *A. baumannii* strains, implying that *A. junii* may be quickly eliminated by the host [5]. The finding that *A. junii* LUH5851 did not survive in our pneumonia model supports this presumption, although experiments focusing on the first hours of infection are necessary to assess the relationship between clearance and the innate immune response. Multiple factors play a role in clearance of pathogens from the lungs, including phagocytosis and killing by neutrophils and macrophages, by antimicrobial peptides and serum components [16]. As we used neutropenic mice, other factors than neutrophils contributed to the rapid clearance of the *A. junii* strain and RUH3023^{T}. It is of note that RUH3023^{T} was more susceptible to killing by human serum in vitro than *A. baumannii* RUH875, RUH134, LUH5875 and LUH8326 (de Breij et al, unpublished).The type strain of *A. baumannii* (ATCC19606^{T}) was used as a model strain in several virulence studies [9,17–21]. The finding that this strain, in contrast to other *A. baumannii* strains, did not survive in the mouse pneumonia model and in a mouse thigh infection model [22] (de Breij et al, unpublished), challenges the relevance of this strain as representative for the *A. baumannii* species. Altogether, noted differences in virulence among *A. baumannii* strains, as also observed by others [15,23], underscore that the choice of strain is a critical variable in virulence studies. The reference strains of EU clones I and II that are associated with outbreaks worldwide were highly virulent in our study. It is important to further assess whether this is a general feature of these clones as this might have implications for clinical diagnostics [24,25].

Eveillard et al [15] described a significant increase in TNFα and MIP-2 levels in lungs of mice after infection with five different *A. baumannii* strains. They showed that MIP-2 levels were higher in mice after the second day of infection with two virulent strains than with three less-virulent strains. Further to this, we found a clear association with the severity of infection and levels of the anti-inflammatory cytokine IL-10. The effects of IL-10 during bacterial infections are complex. During an overwhelming infection, as in our mouse studies, the anti-inflammatory effects of IL-10 are most likely beneficial to the host by down-regulating inflammation and its unfavourable effects [26,27]. However, IL-10 also hampers the appropriate pro-inflammatory response to the bacteria, and then it can be hazardous for the host [28,29]. Indeed, we also found that low levels of IL-12p40 and IL-23 were associated with a poor outcome, which is in agreement with Happel et al, who

demonstrated the critical roles of IL-12p40 and IL-23 in host survival in a murine model of *Klebsiella pneumoniae* infection [30]. Others reported that increased levels of IL-12p40 as well as TNFα and IL-4 in neutropenic mice infected with *Cryptococcus neoformans* were associated with survival of these mice but not with a decreased fungal burden [31]. IL-23 is a cytokine together with enhanced IL-1β and IL-6 production known to drive an IL-17-producing T cell population in mice [32] that enhance epidermal defence and neutrophil influx. 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 [23].

In conclusion, a striking difference in morbidity and mortality associated with *A. baumannii* strains was noted, with EU clone I and II strains being the most virulent. Furthermore, the outcome of experimental *A. baumannii* pneumonia is associated with IL-10 and IL-12p40/IL-23 levels. Future studies will have to clarify whether this response influences the impact of *A. baumannii* strains in the human host. If so, levels of these mediators may have predictive values or be targets for treatment.

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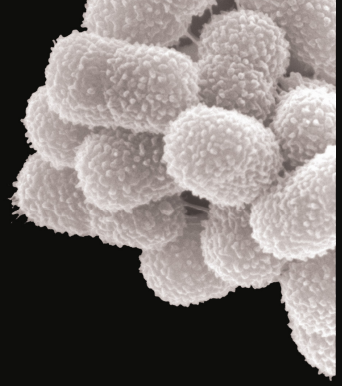

Chapter 5

The clinical success of *Acinetobacter* species; genetic metabolic and virulence attributes

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Abstract

An understanding of why certain *Acinetobacter* species are more successful in causing nosocomial infections, transmission and epidemic spread in healthcare institutions compared with other species is lacking. We used genomic, metabolomic and virulence studies to identify differences between *Acinetobacter* species. Fourteen strains representing nine species were examined. Genomic analysis of six strains showed that the *A. baumannii* core genome contains many genes important for diverse metabolism and survival in the host. Few were unique compared to less clinically successful species. In contrast, when the accessory genome of an individual *A. baumannii* strain was compared to a less successful species (*A. calcoaceticus*), many unique operons with putative virulence function were identified, including the *csu* operon, the acinetobactin chromosomal cluster, and bacterial defence mechanisms. Metabolomic studies showed that compared to *A. calcoaceticus* (RUH2202), *A. baumannii* ATCC 19606^T was able to utilise nitrogen sources more effectively and was more tolerant to pH, osmotic and antimicrobial stress. Virulence differences were also observed, with *A. baumannii* ATCC 19606^T, *A. pittii* SH024, and *A. nosocomialis* RUH2624 persisting and forming larger biofilms on human skin than *A. calcoaceticus*. *A. baumannii* ATCC 19606^T and *A. pittii* SH024 were also able to survive in a murine thigh infection model, whereas the other two species were eradicated. The current study provides clues to the elucidation of differences in clinical relevance among *Acinetobacter* species.

Introduction

In contemporary medicine, certain *Acinetobacter* species have proven to be highly successful in their ability to cause outbreaks and develop antibiotic resistance [1,2]. However, great diversity exists in the clinical importance of the various species, with some being dominant as human pathogens and others merely acting as colonizing or environmental organisms [2]. To date, with the recent description of the novel species *Acinetobacter pittii* (former name *Acinetobacter* genomic species [gen. sp.] 3) and *Acinetobacter nosocomialis* (former name *Acinetobacter* gen. sp. 13TU) [3], the genus *Acinetobacter* comprises 26 validly named species and 9 DNA–DNA hybridization groups (gen. sp.) with provisional designations. *A. baumannii* has long been considered the most clinically important species, with the greatest number of healthcare–related outbreaks and reports of multidrug resistance. More recently, and likely as a consequence of improved laboratory identification, *A. pittii* and *A. nosocomialis* have also surfaced as clinically significant, with increasing reports of outbreaks and antibiotic resistance [4,5,6,7,8,9]. Species that have less commonly been associated with human disease include *A. lwoffii*, *A. junii*, and *A. haemolyticus*, and some species have only been identified as colonizing human skin or very rarely described as causing human disease, such as *A. johnsonii* and *A. radioresistens* [2,7]. To our knowledge, *A. calcoaceticus* has never been implicated in serious human disease [2]. However, given the difficulty in phenotypically differentiating it from *A. baumannii*, *A. pittii* and *A. nosocomialis*, these species are often grouped together in diagnostic microbiology laboratories as the '*A. calcoaceticus – A. baumannii* complex'.

Thus far, the attributes that make one *Acinetobacter* species more adept at causing human outbreaks and disease than another are poorly understood. Previous studies have shown that *A. baumannii* has the ability to survive in both wet and dry conditions in the hospital environment [10,11,12]. A recent clinical study showed that relative to *A. nosocomialis*, *A. baumannii* was an independent predictor of mortality [5]. A variety of virulence mechanisms have been identified in *A. baumannii*, including siderophore–mediated iron acquisition systems, biofilm formation, adherence and outer membrane protein function, the lipopolysaccharide (LPS), capsule formation, and quorum–sensing [13]. Significantly less is understood about the non–*baumannii* species. In this study, we used a systems biology approach involving genomics, phenomics and virulence studies, to identify species characteristics that may explain why some *Acinetobacter* species are successful as human pathogens and others are not. This work deepens our understanding of *Acinetobacter* biology, which is critical to develop improved

diagnostic, preventative and therapeutic strategies against this troublesome human pathogen.

Materials and Methods

Bacterial strains and culture conditions

The 14 strains included in this study are shown in Table 1. The genomes of eight strains were sequenced in the present investigation, while for six strains, the publicly available genomes were used (Table 1). Cultures were performed at 30°C or 37°C on sheep blood agar plates (bioMérieux, Boxtel, The Netherlands) or in Luria–Bertani (LB) broth.

Whole genome sequencing

Genomic DNA was extracted using the Invitrogen Easy–DNA kit (Invitrogen, CA, USA) or as described by Boom et al. [32]. Genomes were sequenced using 454 FLX pyrosequencing (Roche) with DNA standard fragment and 3 kb jumping libraries according to the manufacturer's recommendations [33]. Genomes were assembled using Newbler and the runAssembly script was then used to assemble reads into contigs. Final assemblies were BLASTed to the NCBI non-redundant database and UniVecCore was used to remove any contaminating sequence. For annotation, *ab initio* gene models were predicted using GeneMark, Glimmer3, MetaGene, and Zcurveb (Delcher et al, Nucleic Acids Res 27, 1999; Guo et al, Nucleic Acids Res 27, 2003; Noguchi et al, Nucleic Acids Res 34, 2006). An evidence-based approach constructed open reading frames (ORFs) from BLASTX hits with the NCBI non–redundant protein database; all BLAST hits with e–values better than 1 x 10^{-10} were used as BLAST evidence. A summary of gene finding data for each locus can be viewed at the Broad Institute *Acinetobacter* group database (http://www.broadinstitute.org/annotation/genome/Acinetobacter_group/GenomeStats.html).

Phylogenetic analysis and comparative genomics

Predicted proteins from each *Acinetobacter* genome were compared using an all–against–all BLAST search and *Pseudomonas aeruginosa* PAO1 was used as the outgroup. Reciprocal best blast matches (RBM), regardless of percent identity, were stored in a custom MySQL relational database to facilitate identification of orthologous groups shared by selected phylogenetic and phenotypic groups of organisms. RBM matching proteins were clustered using the Markov clustering algorithm implemented in MCL [34], and clusters with one protein per genome were defined. These represent orthologous core genes that are

present as a single copy in each genome. The protein sequences for each cluster were aligned using CLUSTALW [35] and the resulting multiple sequence alignments were concatenated for tree building. A neighbour joining (NJ) tree was made using MEGA4 [36] and evaluated using 100 bootstrap replicates. The criteria used to define an operon were (*i*) genes were consecutive, (*ii*) genes were transcribed in the same orientation, (*iii*) the intergenic distance between the genes was no longer than 150 bp, and (*iv*) gene length was at least 450 bp [37,38].

Table 1. Characteristics of bacterial strains used in this study

Species	Strain	Origin (Place, year)	Source	Genome Size (Mb)	No. of Genes	Reference	Genbank Accession No.
A. baumannii [1]	ATCC 19606[T]	Unknown, before 1949	Urine	3.97	3766	This study	ACQB00000000
A. baumannii	ATCC 17978	Unknown, ~1951	Unknown	3.98	3791	[45]	CP000521
A. baumannii	AB0057	Washington, D.C., USA,	Blood	4.05	3853	[46]	CP001182
A. baumannii	AB307–0294	Buffalo, NY, USA, 1994	Blood	3.76	3458	[46]	CP001172
A. baumannii	AYE	Le Cremlin–Bicêtre, FR, 2001	Urine	3.94	3607	[47]	CU459141
A. baumannii	ACICU	Rome, IT, 2005	CSF	3.90	3667	[48]	CP000863
A. calcoaceticus [1]	RUH2202	Malmoe, SE, 1980–82	Wound	3.88	3566	This study	ACPK00000000
A. pittii [1]	SH024	Cologne, DE, 1993	Skin	3.97	3689	This study	ADCH0000000
A. nosocomialis [1]	RUH2624	Rotterdam, NL, 1987	Skin	3.87	3631	This study	ACQF00000000
A. lwoffii	SH145	Cologne, DE, 1994	Skin	3.48	3134	This study	ACPN0000000
A. junii	SH205	Cologne, DE, 1994	Skin	3.46	3186	This study	ACPM0000000
A. radioresistens	SH164	Cologne, DE, 1994	Skin	3.16	2874	This study	ACPO0000000
A. johnsonii	SH046	Cologne, DE, 1994	Skin	3.69	3363	This study	ACPL00000000
A. baylyi	ADP1	Atlanta, GA, USA, before 1958	Soil	3.60	3325	[49]	CR543861

DE, Germany; FR, France; IT, Italy, NL, The Netherlands; SE, Sweden; US, United States
[1]Representative strains of the *A. calcoaceticus-A. baumannii* complex that were analysed in detail.

Metabolic profiling

To assess for metabolic differences between *Acinetobacter* species, we used Phenotype Microarrays (PM) as described previously by Biolog Inc. (Hayward, CA, USA) [39]. This technology uses tetrazolium violet irreversible reduction to formazan as a reporter of active metabolism. Twenty 96–well microarray plates were used (PM 1-20) comprising 1920 different metabolic and toxic compound conditions, including 192 assays of C-source metabolism (PM 1–2), 384 assays of N–source metabolism (PM 3, 6 – 8), 96 assays of P-source and S–source metabolism (PM 4), 96 assays of biosynthetic pathways (PM 5), 96 assays of ion effects and osmolarity (PM 9), 96 assays of pH effects (PM 10), and sensitivity to 240 chemicals (PM 11–20) (Bochner, FEMS Microbiol Rev 33, 2009). In brief, a standardized bacterial cell density suspension containing a tetrazolium redox dye (measures cell respiration) was transferred into wells of the microplates, which contained dried nutrients or chemicals to create the unique culture conditions. Plates were incubated at 37°C and metabolic activity was measured colorimetrically using the redox dye (Bochner, FEMS Microbiol Rev 33, 2009). Bioinformatic software (OmniLog V. 1.5) quantified metabolism as a color–coded kinetic graph. Two independent experiments

were performed for all analyses. A best blast hit approach was used to map all the proteins in the four genomes to the KEGG reactions database using the KEGG proteome database (http://www.genome.jp/kegg/, release 54.1); an E–value threshold of $1x10^{-50}$ was applied. Phenotype microarray data was analyzed using GenoPhenomicon: the activity of each well was predicted as *active/not active* using a support vector machine (SVM) predictor built using SVMpython v.2 (http://www.tfinley.net /software/svmpython2/), using a training dataset of 800 manually validated samples. Relationships between genome content and phenotype microarray data were then inspected.

The analysis of PM data was carried out on the raw data–set provided by Biolog Inc., obtained by three replicates of each substrate. Binary coefficients (1/0) for positive metabolism (1) or no metabolic activity (0) were attributed to each PM well and a matrix of binary vectors, each representing a single *Acinetobacte*r species, was prepared as previously described [40]. Binary data were then used to compute a similarity matrix by using Jaccard coefficient with the software PAST [41].

Growth on human skin equivalents

Human keratinocytes were isolated from fresh mamma reduction surplus skin as previously described [42]. Human epidermal skin constructs were generated as described previously [51]. In brief, human epidermal skin constructs were incubated with 300 µl of a mid–logarithmic bacterial suspension ($3x10^5$ colony forming units [CFU]/ml) at 37ºC (7.3% CO_2). After 1 h, skin constructs were washed with phosphate buffered saline (PBS) to remove non–adherent bacteria and were incubated for an additional 23 h and 47 h. Two circular biopsies (4 mm in diameter) were taken from the skin, homogenized in PBS and serially diluted to determine the number of CFU. A third biopsy of each skin construct was fixed in 4% formaldehyde, dehydrated and embedded in paraffin for subsequent staining with Alcian–blue PAS (Merck, Darmstadt, Germany) for morphological analysis. Three independent experiments were performed.

Bronchial epithelial cell adhesion and cytokine production

Adherence of bacteria to human bronchial epithelial cells (H_{292} cells, ATCC CRL–1848, Manassas, VA, USA) and cytokine production by these cells was determined as described previously [30,43]. In brief, H_{292} cells were incubated for 1 h at 37°C with $1x10^8$ CFU of an overnight bacterial culture on blood agar. Bacterial adherence to H_{292} cells was quantified by light microscopy and the average number of bacteria per 100 epithelial cells was recorded. Two independent experiments were performed in duplicate. For cytokine production, H_{292} cells were washed five times after 1 h of bacterial infection (as described

above) with prewarmed PBS, and fresh RPMI medium was added. After 23 h incubation at 37°C, supernatants were collected and stored at –20°C until determination of cytokine levels. RPMI medium alone was used as a control. Interleukin (IL)–6 and IL–8 were determined by enzyme–linked immunosorbent assays (ELISA, Biosource, CA, USA) according to the manufacturer's instructions. The lower limit of detection was 15 pg/ml for IL–6 and 7 pg/ml for IL–8. Three independent experiments were performed.

Murine thigh infection model

The survival of *Acinetobacter* strains in a mouse thigh muscle infection model was assessed as previously described [44], with modifications. Female Swiss mice (Charles River Nederland, Maastricht, The Netherlands) were made transiently neutropenic by intraperitoneal injection with cyclophosphamide (150 mg/kg body weight in 150 µl) on day 4 and 3 prior to infection. Approximately $1x10^4$ CFU (in 50 µl of saline) of a mid–logarithmic culture was injected in the right thigh muscle (three animals per strain). At 48 h after infection, mice were sacrificed and infected thigh muscles were removed and homogenized in 1 ml PBS and viable counts were performed. The animal studies were approved by the Leiden Experimental Animal Committee (Permit number: 10038) and were performed in compliance with Dutch laws related to the conduct of animal experiments. All efforts were undertaken to minimize suffering.

Statistical analysis

All data were analysed for statistical significance using the Wilcoxon rank sum test. *P* values of ≤ 0.05 were considered statistically significant.

Results

Genome characteristics of the *Acinetobacter* species

As shown in Table 1, 14 genomes were included in this analysis, covering nine different *Acinetobacter* species (species names will be used for non–*baumannii* species throughout). Eight strains were sequenced as part of this study with mean coverage of 22–fold. Overall, the species that make up the *A. calcoaceticus* – *A. baumannii* complex had the largest genomes, with *A. radioresistens* having the smallest (3.16 Mb). Genome sizes of strains within the *A. baumannii* species varied by up to 289 Kb. The number of genes corresponded to genome size, ranging from 3,690 in *A. baumannii* to 2,874 in *A. radioresistens*. Phylogenetic analysis showed that the species that make up the *A. calcoaceticus* – *A. baumannii* complex were most closely related, with *A. nosocomialis*

being closest to *A. baumannii*, followed by *A. pittii* and *A. calcoaceticus* (Figure S1). The other species formed distinct phylogenetic branches (Figure S1).

Analysis of the *A. baumannii* core genome

To understand the genetic core of *A. baumannii*, we first analysed the orthologous genes found in all six *A. baumannii* genomes. This analysis yielded 2,800 genes, indicating that the accessory genome varied between 658 – 1,053 genes depending on the strain. A distribution of the *A. baumannii* core genome based on functional gene categories is shown in Figure 1. Apart from genes of general or unknown function, genes related to molecule transport and metabolism were most abundant (35%), including amino acid (11%), carbohydrate (5%), lipid (5%), nucleotide (3%), coenzyme (4%) and inorganic ion (7%) processing.

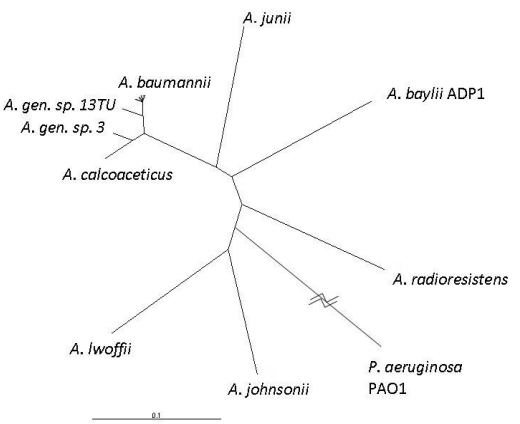

Figure S1. Phylogenetic analysis of the eight sequenced strains of *Acinetobacter* species from this study.

Interestingly, despite *Acinetobacter* deriving its name from akineto meaning non–motile, *A. baumannii* has several core cell motility genes. These include a type IV pilus apparatus and pilus assembly genes (*pilB*, *pilW*, *pilL*, *pilJ*, *pilI*, *pilY1*, *pilQ*, *pilO*, *pilN*, *pilM*), fimbrial biogenesis genes (*fimT*, *pilZ*), and twitching motility genes (*pilU*, *pilT*, which are important for pilus retraction). In fact, it has recently been shown that *A. baumannii* is motile under certain conditions [14,15], and this may play an important role in its ability to colonize and spread on surfaces, and to form biofilms [16]. We also identified 24 genes that appear to determine *A. baumannii* cell shape and division, including the cell division genes (*ftsA*, *ftsZ*, *ftsW*, *ftsK*), the rod shape determining genes (*mreB*, *rodA*) and the *min* operon (*minE*, *minD*, *minC*), which controls the position in which FtsZ assembles in the center of the cell.

Given that cell division biosynthetic machinery has been targeted in other successful antibacterial therapies, the identification of these genes is promising [17].

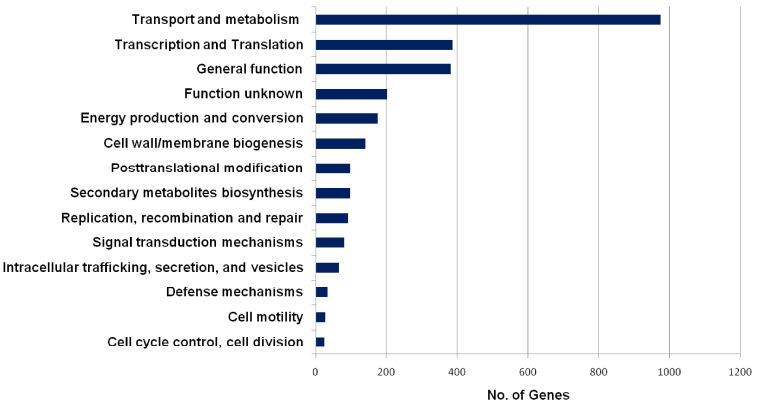

Figure 1. *A. baumannii* core genome. Functional distribution of the genes found in all six *A. baumannii* strains included in this study.

Comparison of *A. baumannii* genome with other *Acinetobacter* species

To begin to decipher the genetic attributes that may help explain why some *Acinetobacter* species are clinically more significant than others, we assessed for genes observed uniquely in pathogenic species of *Acinetobacter* (the six *A. baumannii* genomes, and the *A. pittii* and *A. nosocomialis* genome). This analysis identified 51 genes, including 12 operons, shared among these eight genomes that were not present in the other species (Table S1). Importantly, one of these operons was the *csu* operon, which includes six genes, and codes for proteins involved in a chaperone – usher pili assembly system [18]. This operon appears important for pili assembly, adherence to abiotic surfaces and biofilm formation [18]. The presence of this operon only in pathogenic species of *Acinetobacter* highlights its potential role in determining the clinical success of these species. The predominant functional categorization of the remaining genes was in molecule transport and metabolism, and transcription (Table S1).

Comparison of specific strains of the *A. calcoaceticus – A. baumannii* complex

The data presented thus far provide some evidence that a small number of core genes may partly explain the clinical success of certain *Acinetobacter* species; however the number of genes that differed was few, suggesting that additional genetic characteristics that distinguish pathogenic from less pathogenic strains may be found among the accessory genomes.

Table S1. Unique genes found in pathogenic species of *Acinetobacter* (*A. baumannii* [six strains], *A. pittii* and *A. nosocomialis*) and not in other less or non-pathogenic species

ATCC19606	Description	COG Description	Operon	Homologues (nt ID %)	
				A. pittii	*A. nosocomialis*
ACIB1v1_040004	transcriptional regulator, TetR family	Transcription	No	89.6	90.1
ACIB1v1_050001	CsuA/B	Cell motility		99.4	99.4
ACIB1v1_050002	CsuA	Cell motility		84.1	85.2
ACIB1v1_050003	CsuB	Cell motility	Yes	89.5	87.4
ACIB1v1_050004	CsuC	Cell motility		96.3	94.5
ACIB1v1_050005	CsuD	Cell motility		95.8	95.3
ACIB1v1_050006	CsuE	Cell motility		95.7	99.3
ACIB1v1_050012	transcriptional regulator, TetR family	Transcription	No	72.2	72.2
ACIB1v1_050037	acetyltransferase, gnat family	General function prediction only	No	96.0	99.3
ACIB1v1_090011	transcriptional regulator, AsnC family	Transcription	No	95.3	97.3
ACIB1v1_090012	kynureninase	Amino acid transport and metabolism		90.6	95.9
ACIB1v1_090013	proline-specific permease ProY	Amino acid transport and metabolism	Yes	90.2	96.8
ACIB1v1_090014	conserved hypothetical protein	Lipid transport and metabolism		72.0	83.8
ACIB1v1_090017	conserved hypothetical protein	Function unknown	No	79.6	89.0
ACIB1v1_150007	transcriptional regulator, GntR family	Transcription	Yes	99.2	100.0
ACIB1v1_150008	dihydroxy-acid dehydratase	Carbohydrate transport and metabolism		99.5	99.5
ACIB1v1_150009	MFS family permease	General function prediction only	Yes	99.8	99.0
ACIB1v1_150010	hypothetical protein	Function unknown		97.3	99.1
ACIB1v1_150012	conserved hypothetical protein	No COG annotation	No	92.9	97.7
ACIB1v1_150014	oxidoreductase FMN-binding	Energy production and conversion	Yes	97.8	97.6
ACIB1v1_150015	saccharopine dehydrogenase	Function unknown		95.8	96.6
ACIB1v1_150017	transcriptional regulator, MerR family	Transcription	No	91.3	97.8
ACIB1v1_150046	transcriptional regulator, LysR family	Transcription	No	97.1	97.7
ACIB1v1_150047	hypothetical protein	Function unknown	Yes	97.9	95.1
ACIB1v1_150048	hypothetical protein	Function unknown		97.4	96.0
ACIB1v1_150094	putative acid phosphatase	General function prediction only	Yes	97.8	97.2
ACIB1v1_150095	conserved hypothetical protein	No COG annotation		93.0	94.4
ACIB1v1_230050	transcriptional regulator, LysR family	Transcription	No	96.7	95.8
ACIB1v1_230051	3-oxoacyl-[acyl-carrier-protein] reductase	Lipid transport and metabolism	No	95.1	95.1
ACIB1v1_240001	conserved hypothetical protein	General function prediction only	No	87.5	93.6
ACIB1v1_240049	FAD dependent oxidoreductase	Amino acid transport and metabolism	Yes	94.8	92.5
ACIB1v1_240050	ABC transporter, permease	Inorganic ion transport and metabolism		97.7	98.8
ACIB1v1_240051	ABC transporter, ATP binding protein	Amino acid transport and metabolism		95.5	97.6
ACIB1v1_240052	2-aminoethylphosphonate ABC transport system, 1-amino-ethylphosphonate-binding protein component	Inorganic ion transport and metabolism	Yes	98.3	98.8
ACIB1v1_240053	transcriptional regulator, GntR family	Transcription		97.9	99.6
ACIB1v1_240056	acyl carrier protein phosphodiesterase	Lipid transport and metabolism	No	96.0	97.5
ACIB1v1_240122	cyanate transport protein CynX	Inorganic ion transport and metabolism	Yes	78.8	90.0
ACIB1v1_240123	guanine deaminase	Nucleotide transport and metabolism		80.6	91.3
ACIB1v1_240159	glutathione-dependent formaldehyde dehydrogenase	Amino acid transport and metabolism	No	97.7	96.9
ACIB1v1_240186	4-hydroxybenzoate transporter	Amino acid transport and metabolism	No	94.5	94.5
ACIB1v1_250041	cis,cis-muconate transport protein	Amino acid transport and metabolism	No	98.9	98.6
ACIB1v1_250167	conserved hypothetical protein	No COG annotation	No	96.2	94.2
ACIB1v1_250168	transcriptional regulator, LysR family	Transcription	No	98.2	98.6
ACIB1v1_250169	tartrate dehydrogenase	Energy production and conversion	Yes	98.9	98.7
ACIB1v1_250170	betaine/choline/glycine transport protein	Cell wall/membrane/envelope biogenesis		99.3	99.5
ACIB1v1_250174	putative dioxygenase subunit beta	Energy production and conversion		95.6	97.3
ACIB1v1_430020	transcriptional regulator, TetR family	Transcription	No	93.1	93.6
ACIB1v1_460007	Peptidase M20D, amidohydrolase	General function prediction only	No	93.2	92.1
ACIB1v1_490004	TonB-dependent receptor	Inorganic ion transport and metabolism	Yes	96.2	96.8
ACIB1v1_490005	aminopeptidase N	Amino acid transport and metabolism		97.6	97.4
ACIB1v1_820017	haloacid dehydrogenase	General function prediction only	No	93.9	90.6

To interrogate the accessory genome in more detail, we analysed representative strains from the four species that make up the *A. calcoaceticus* – *A. baumannii* complex (Table 1). A distribution of genes is shown in Figure 2A. A total of 2505 genes were common to all four species, with the greatest number of unique genes observed in *A. baumannii* ATCC 19606^T. Based on category of gene (COG) functional classification, the distribution of genes unique to *A. baumannii* ATCC 19606^T compared to each one of the other three

strains was similar, with the greatest number of unique genes having a role in amino acid, carbohydrate and lipid transport and metabolism, and transcription (Figure 2B).

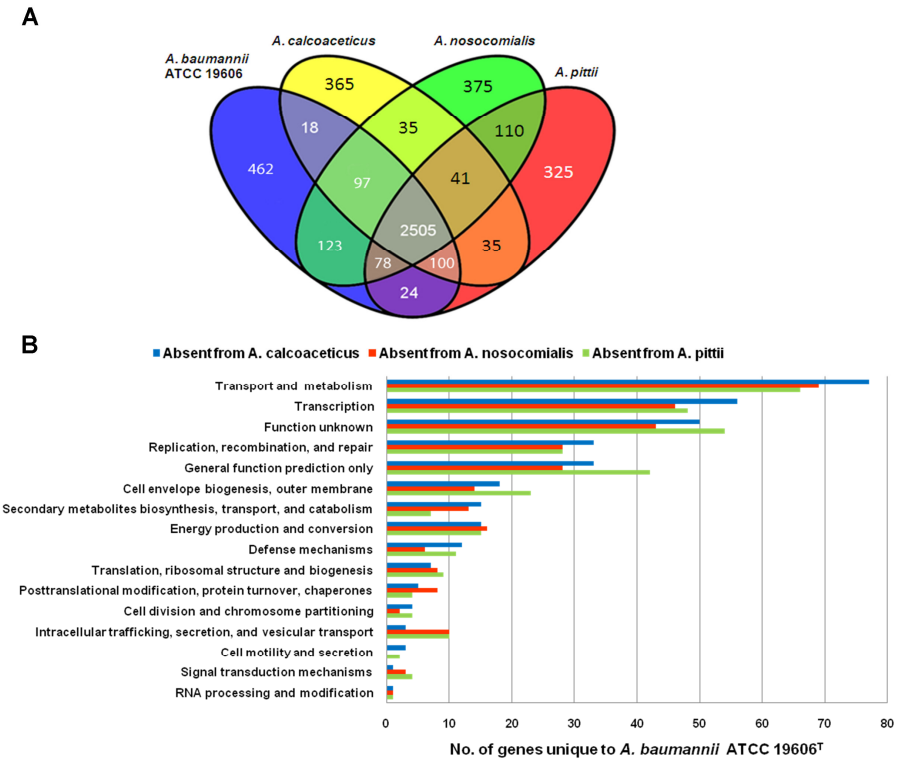

Figure 2. Distribution of genes in individual strains of the *A. calcoaceticus* – *A. baumannii* complex. (**A**) Venn diagram showing the number of overlapping genes between the four strains that make up the *A. calcoaceticus* – *A. baumannii* complex. (**B**) The number of genes present in *A. baumannii* ATCC 19606^T but absent in each of *A. calcoaceticus*, *A. pittii* and *A. nosocomialis*.

Of most interest was the comparison between *A. baumannii* ATCC 19606^T and *A. calcoaceticus*. This comparison identified 759 unique genes in *A. baumannii* ATCC 19606^T. Of these, only 169 were found in the other five *A. baumannii* genomes analysed in this study, indicating that the majority of the unique genes (78%) were part of the accessory genome of *A. baumannii* ATCC 19606^T. Of the 759 unique genes, 333 had a COG classification, and they were significantly overrepresented in several functions necessary for basic bacterial growth and survival, including transcription (56 genes), DNA replication, recombination, and repair (33 genes), amino acid, inorganic ion and carbohydrate

transport and metabolism (66 genes), and cell envelope biogenesis and outer membrane function (19 genes).

Of the 333 genes, there were 69 unique operons that were enriched in virulence–related genes, including those involved in siderophore transport and biosynthesis, LPS biosynthesis, pili and biofilm formation, Curli fimbriae assembly, and bacterial defence mechanisms (Table 2). Several operons responsible for iron handling were identified, including the acinetobactin chromosomal locus (operons 36 – 39, Table 2), encoding a key *Acinetobacter* siderophore [19,20]. The genetic organisation of this locus and homologues in *A. pittii*, *A. nosocomialis* and *A. calcoaceticus* are shown in Figure 3A. *A. calcoaceticus* and *A. nosocomialis* lacked the full complement of genes that make this locus (Table 2 and Figure 3A). We also identified a more recently described siderophore operon (operon 17, Table 2 and Figure 3B) [14], made up of eight genes, with *A. baumannii* ATCC 19606^T being the only strain with the full complement of genes, and *A. nosocomialis* and *A. calcoaceticus* being deficient in most of them.

Table 2. Select operons with putative virulence function found in *A. baumannii* ATCC 19606^T and not in *A. calcoaceticus*

Operon ID	Function	Genes	*A. baumannii* ATCC 19606^T ORFs[1]	Homologues average similarity (ID% ± SD)[2]	
				A . pittii	*A. nosocomialis*
2	Pili assembly and biofilm form.	*csuAB/ABCDE*	ACIB1v1_50001–6	91.8 ± 7.1	91.8 ± 7.1
17	Siderophore transp. bios.	*cirA* , *menG* , *iucA/C, araJ* , *rhbE/C*	ACIB1v1_160094–101[3]	46.2 ± 17.9	31.1 ± 3.7
29	Defense mechanism	*cas1, csy1, csy2, csy3, csy4*	ACIB1v1_260071–75	–	–
36	Siderophore transp. bios.	*bauD* , *bauC* , *bauE* , *bauB* and *bauA*	ACIB1v1_480066–70	97.8 ± 1.0	–
37	Siderophore transp. bios.	*basC* and *basD*	ACIB1v1_480071–72	97.4 ± 1.0	–
38	Siderophore transp. bios.	*basE, basF, basG*	ACIB1v1_480073–75	97.6 ± 0.3	47.8 ± 0.4
39	Siderophore transp. bios.	*barA, barB, basH, basI, basJ*	ACIB1v1_480076–80	95.1 ± 6.3	32.8 ± 0.8
40	Siderophore transp. bios.	*tonB* , PEPN	ACIB1v1_490004–5	97.0 ± 0.3	97.5 ± 0.3
46	Cell motility and secretion	*pilA*	ACIB1v1_560044–45	60.2 ± 14.5	73.3 ± 0.3
47	LPS biosynthesis	*lpsC* and *lpsE*	ACIB1v1_600015–16[4]	33.9	68.2 ± 29.8
50	Curli fimbriae assembly	*csgG*	ACIB1v1_700078–80	94.8 ± 1.1	–
56	LPS biosynthesis	*wzx* , *degT* , *wbbJ* , *mviM* and *vipA*	ACIB1v1_740018–22[6]	42.6 ± 23.8	48.3 ± 17.2

Bios., Biosynthesis; Form., Formation; ID, Identity; SD, Standard Deviation; Transp., Transport.
[1]Based on Microbial Genome Annotation Platform (www.cns.fr/agc/mage) [50]. [2]Expressed as the average identity at the nucleotide level ± standard deviation. [3]Only three, two and two genes (out of eight) are found in *A. pittii*, *A. nosocomialis* and *A. calcoaceticus*, respectively. The homologues identified exhibited low similarity. [4]Both genes belong to an LPS operon that spans from ORF ACIB1v1_600009 to 16, and which is only partially present within *A. calcoaceticus* (three of eight genes are absent). [5]*lpsC* is absent from *A. pittii* genome. [6]Only *vipA* is present in *A. calcoaceticus* and exhibited moderate similarity. The operon is poorly conserved and partially present also in *A. pittii* and *A. nosocomialis*.

Unique genes related to bacterial defence mechanisms were also observed in *A. baumannii* ATCC 19606^T, including those coding for ABC transporters, and CRISPR – (Cas) and phage–related proteins. Clustered regularly interspaced short palindromic repeats (CRISPRs) are recently described adaptive bacterial immune mechanisms that protect bacteria from invading foreign genetic elements such as bacteriophages [21,22]. Such systems are likely advantageous in hostile environments, and when combined with other

phage resistance mechanisms, may provide a survival benefit to the bacterial host [21]. The *A. baumannii* ATCC 19606^T CRISPR system includes *cas1* and *cas3*; however we could not locate *cas2*, which is thought to be required with *cas1* to form a functional CRISPR system [22]. The CRISPR operon was not found in *A. calcoaceticus*, *A. nosocomialis* or *A. pittii*.

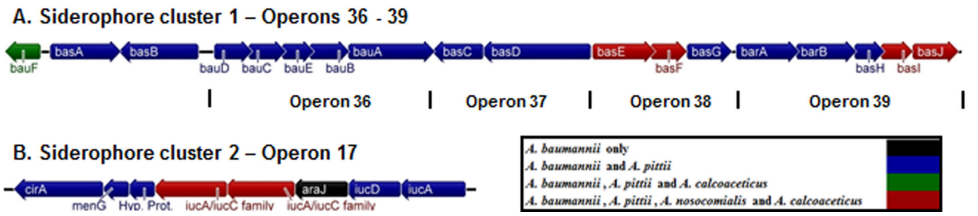

Figure 3. Genetic organisation and conservation of the siderophore clusters found in *A. baumannii* ATCC 19606^T and not in *A. calcoaceticus*. (A) Siderophore cluster 1 (operons 36 – 39) is known as the acinetobactin chromosomal cluster, and (B) siderophore cluster 2 (operon 17) (See Table 2 for details about the operons). The presence of homologues for each gene in *A. pittii*, *A. nosocomialis*, and *A. calcoaceticus* is shown.

Comparison of the metabolic versatility of specific strains of the *A. calcoaceticus* – *A. baumannii* complex

Given the predominance of metabolism genes differentiating pathogenic and less pathogenic strains, we analyzed the metabolic profile of the four species of the *A. calcoaceticus* – *A. baumannii* complex using phenotype microarrays. Of the 1920 conditions tested, the four species shared 1356 metabolic responses (70.6%), of which 795 compounds or conditions could be utilized by all the species and 561 by none of them. A summary of the entire metabolic profile of the four species is shown in Figure 4. *A. baumannii* ATCC 19606^T appeared to utilize peptide nitrogen sources (PM 6–8) more effectively and to be more tolerant to pH stress (PM 10) than the other three species. *A. baumannii* ATCC 19606^T and *A. pittii* had a reduced ability to utilise most of the phosphorus and sulfur sources (PM 4) (Figure 4).

We then focused on the most clinically disparate of the species, and compared *A. baumannii* ATCC 19606^T with *A. calcoaceticus* in more detail. In 195 conditions, *A. baumannii* ATCC 19606^T was significantly more metabolically active than *A. calcoaceticus*. These conditions comprised 10 carbon sources, 105 nitrogen sources (of which 98 were di– and tri–peptides) and 80 stress conditions, of which 26 related to osmotic and pH stress and 54 related to the presence of antimicrobials and other cytotoxic compounds.

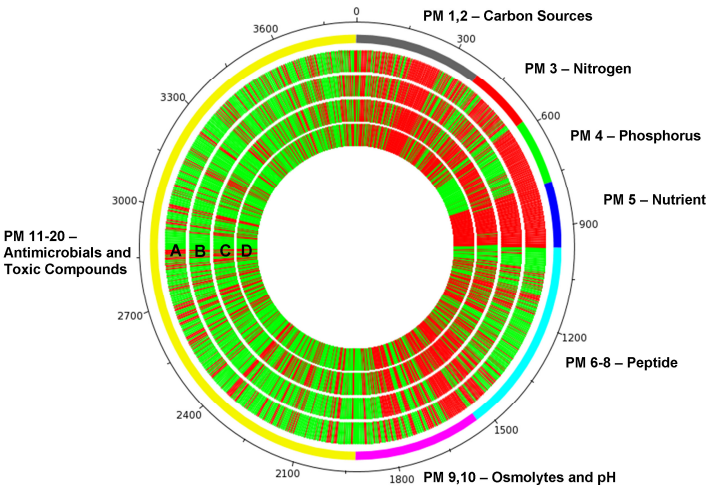

Figure 4. Metabolic diversity of specific strains of the *A. calcoaceticus – A. baumannii* complex. Phenotype Microarray (PM) comparative results showing the number of compounds used (green) or not used (red) by *A. baumannii* ATCC 19606^{T} [A], *A. nosocomialis* [B], *A. pittii* [C] *and A. calcoaceticus* [D]. The external circle and PM number represent the Biolog plate number.

Apart from the likely survival advantage inferred by the greater ability of *A. baumannii* ATCC 19606^{T} to metabolise in the presence of osmotic, pH and antimicrobial exposure, one of the carbon sources utilized by this strain was D–glucarate. D–glucarate is found in the human body and has been shown to be a carbon source utilized by a range of gram–negative bacteria [23,24]. D–glucarate catabolism generates α–ketoglutarate, which enhances the citric acid cycle. Recently, over–expression of the citric acid cycle was shown to occur in an *A. baumannii* strain with increased virulence in the presence of ethanol [25].

Virulence differences between strains of the *A. calcoaceticus – A. baumannii* complex

Given the differences in the number of operons with putative virulence function that were observed between the four species of the *A. calcoaceticus – A. baumannii* complex, we performed a range of in vitro and in vivo virulence studies to characterise further the functional significance of their genetic differences. Given the predilection of *A. baumannii* to colonise or infect the respiratory tract, we first analysed the interaction of the four species with human bronchial epithelial cells. Cell adherence and pro–inflammatory cytokine response (IL–8) was most pronounced with *A. pittii* ($P < 0.05$) (Figure 5, A, B and C). All strains induced similar levels of IL–6 in these cells.

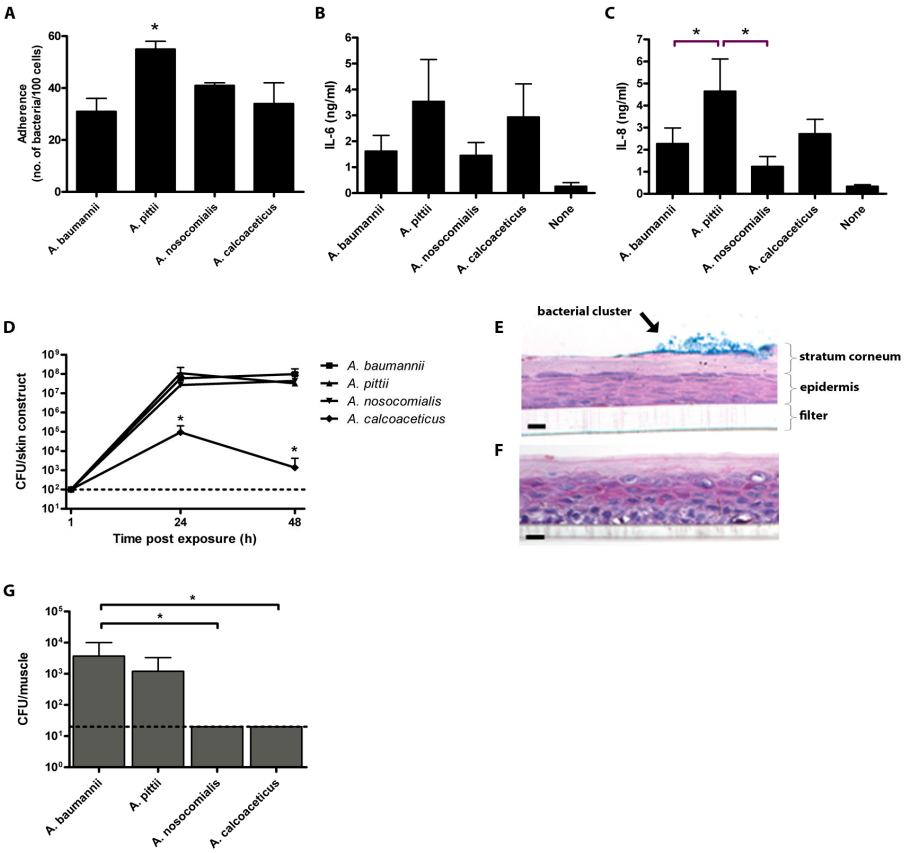

Figure 5. Virulence attributes of individual strains belonging to the *A. calcoaceticus* – *A. baumannii* complex. (A) Adherence of *A. baumannii* ATCC 19606^T, *A. pittii*, *A. nosocomialis* and *A. calcoaceticus* to human bronchial epithelial cells after 1 h. Results are expressed as mean number of bacteria per 100 epithelial cells ± standard deviation (SD) of two independent experiments performed in duplicate. (**B**) Levels of IL–6 and (**C**) IL–8 in the culture medium of human bronchial epithelial cells after 24 hour stimulation with specific strains of the *A. calcoaceticus* – *A. baumannii* complex. Results are expressed as mean levels of IL–6 and IL–8 (in ng/ml) ± SD of three independent experiments. Asterisk signifies statistical significance ($P < 0.05$). (**D**) Persistence and biofilm formation of *A. baumannii* ATCC 19606^T (squares), *A. pittii* (upward triangles), *A. nosocomialis* (downward triangles) and *A. calcoaceticus* (diamonds) on three–dimensional human skin constructs. Results are expressed as mean CFU per skin construct ± SD of three independent experiments. Dotted line represents the lower limit of detection. (**E**) Alcian–blue PAS staining shows biofilm formation (black arrow) on human skin constructs by *A. baumannii* ATCC 19606^T but not by (**F**) *A. calcoaceticus*. Scale bar is equivalent to 20 µm. (**G**) Approximately 1x10^4 CFU were injected in the thigh muscles of neutropenic mice and the number of viable bacteria was determined after 48 h. Results are expressed as mean number of bacteria (in CFU/muscle) ± SD from three animals. Dotted line represents lower limit of detection. Asterisk signifies statistical significance ($P < 0.05$).

Given the likely importance of biofilm formation to the success of *Acinetobacter* in hospitals, we next tested the four species in a unique biofilm assay. Thus far, the correlation between biofilm formation on abiotic surfaces and clinical significance has been poor [26]. Therefore, we used a novel assay that may predict the ability of *Acinetobacter* to colonise and form a biofilm on human skin. Using a three–dimensional human skin construct [51], we observed that *A. baumannii* ATCC 19606^T, *A. pittii* and *A. nosocomialis* were able to multiply rapidly and persist on human skin, whereas *A. calcoaceticus* grew to a significantly lower density (Figure 5D). In addition, biofilms of the former three species were visible on the stratum corneum, whereas no such bacterial structures were seen for *A. calcoaceticus* (Figure 5, E and F). Finally, we assessed the survival of the four strains in a neutropenic mouse thigh muscle infection model. *A. baumannii* ATCC 19606^T and *A. pittii* were able to survive in the thigh muscle at least up to 48 h, whereas *A. calcoaceticus* and *A. nosocomialis* were eradicated (Figure 5G).

Discussion

This study provides a combined genomic, phenomic and virulence assessment of a range of *Acinetobacter* species that have been variably associated with humans. From a genomic analysis of nine different *Acinetobacter* species, we identified a small number of genes unique to pathogenic species. The majority of these genes are predicted to be important for molecule transport and metabolism but also included the putative virulence *csu* operon. Investigating the accessory genome of individual strains of the four species of the *A. calcoaceticus – A. baumannii* complex, we found a range of unique operons with putative functions relating to host survival and virulence in *A. baumannii* ATCC 19606^T but not in *A. calcoaceticus*. *A. pittii* appeared most similar to *A. baumannii* ATCC 19606^T, whereas *A. nosocomialis* lacked several of these important operons, particularly the full repertoire of genes of the acinetobactin chromosomal locus. Global metabolomic studies supported the genomic analysis in that *A. baumannii* ATCC 19606^T was able to utilise more carbon and nitrogen sources, and was more tolerant to a range of cellular stresses than *A. calcoaceticus*. Moreover, the pathogenic species were able to multiply and form biofilms on human skin significantly more than *A. calcoaceticus*. Only *A. baumannii* and *A. pittii* were able to survive in a mammalian thigh infection model.

As a consequence of improved laboratory speciation, it is becoming apparent that non–*baumannii* species, particularly *A. nosocomialis* and *A. pittii*, are clinically significant human pathogens. For example, in a recent study from Norway, *A. nosocomialis* was the most common species (47%) isolated from blood cultures over a three–year period,

followed by *A. pittii* (20%) [27]. With regard to their clinical impact, a more contemporary study has shown that relative to *A. nosocomialis*, bacteremia with *A. baumannii* was an independent predictor of mortality [5]. Interestingly, and consistent with our study findings, there was no significant difference between *A. baumannii* and *A. pittii*, however the number of patients in the *A. pittii* group was small [5]. Genetically and metabolically, we showed that *A. pittii* appeared similar to *A. baumannii*, and they also behaved similarly in the mammalian infection model. *A. nosocomialis* lacked several of the virulence related operons, particularly the acinetobactin siderophore cluster, and its metabolome was more closely aligned to *A. calcoaceticus*, both of which may explain its failure to survive in the murine model and its reduced virulence in clinical studies [5].

Within mammalian hosts, free iron is often a scarce resource and for pathogenic organisms to survive in vivo they often utilize a range of iron scavenging systems. Such systems have been analysed across different *A. baumannii* strains [14,29] however this is the first analysis, to our knowledge, of such genes in non–*baumannii* species. In addition to the acinetobactin chromosomal locus, we observed another siderophore cluster and a putative iron uptake receptor in *A. baumannii* ATCC 19606^T that was not present in *A. calcoaceticus*. This second cluster (operon 17 in Table 2) is a recently described siderophore cluster made up of eight genes that is well conserved across *A. baumannii* strains [14]. The full repertoire of genes from this cluster was not found in *A. pittii*, *A. nosocomialis*, and *A. calcoaceticus*, and the few homologues identified exhibited low similarity (Table 2). Such genetic differences between *Acinetobacter* species in key virulence attributes may help explain why some species have greater clinical impact.

Apart from genes involved in metabolism and transcription, we identified the *csu* operon as an operon found in pathogenic species of *Acinetobacter* (six *A. baumannii* strains, *A. pittii* and *A. nosocomialis*) but not in non–pathogenic species. Loss of function of this operon leads to a lack of pili–like structures on the surface of *A. baumannii* and to loss of cell attachment and biofilms on abiotic surfaces [18]. Interestingly, this operon was not shown to be important for attachment to and cytokine production by human bronchial epithelial cells [30]. We hypothesize that this operon may aid in *Acinetobacter* attachment and colonization of plastic medical devices such as ventilator tubing and catheters, with a subsequent increased risk of invasive infection. The definitive role of this operon in mammalian virulence requires further evaluation.

We observed a diverse repertoire of core metabolic genes in *A. baumannii*, which is likely to be important for its ability to survive in vivo, as well as in unique ecological niches of healthcare institutions. To assess the global metabolic capabilities of the *Acinetobacter* species, we used phenotype microarrays, which enabled us to assess nearly 2000 metabolic and toxic compound conditions. Overall, *A. baumannii* ATCC 19606^T was

able to utilize nitrogen sources more effectively and was more tolerant to pH stress than *A. nosocomialis*, *A. pittii* and *A. calcoaceticus*. The differences were more marked when *A. baumannii* ATCC 19606^T was compared to *A. calcoaceticus*. 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. Several studies have highlighted the key role of the Pho regulon in phosphate management, virulence and stress response [31]. 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.

Taken together, these data provide a systems biology approach to understanding the potential differences between important species of the *Acinetobacter* genus. We provide genetic and metabolic insights into why some species may be more clinically important than others, and also highlight the functional significance of these differences in various virulence models. A limitation of our study is that we only analysed one strain for each of the non-*baumannii* species, and our results need confirmation using a larger set of strains. Furthermore, confirmation of our findings using targeted gene deletion and complementation is required to define the significance and role of the unique operons found in pathogenic versus non-pathogenic species. Overall, these data provide useful insights to the elucidation of differences in clinical relevance among *Acinetobacter* species.

Acknowledgements

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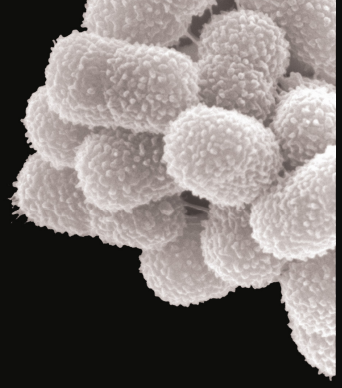

Chapter 6

Cryo electron tomographic analysis of membrane vesicle formation by *Acinetobacter baumannii* ATCC19606^T at different growth stages

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Submitted for publication

Abstract

Acinetobacter baumannii is an important nosocomial pathogen responsible for colonization and infection of critically ill patients. The effect of the bacterium on the host follows from the interplay between virulence attributes of the micro-organism and the condition of the host. As some virulence attributes can be delivered to the host by membrane vesicles, this study aimed at characterization of the formation and morphology of membrane vesicles formed by *A. baumannii* ATCC19606^{T} in vitro using cryo-electron tomography. Results revealed that different membrane vesicles were formed by *A. baumannii* during the various stages of its life cycle: (i) small outer membrane vesicles (OMVs; ±30 nm in diameter) formed at distal ends in the log-phase; (ii) larger OMVs (200-500 nm) formed at septa during cell division; (iii) inner and outer membrane vesicles (IOMVs; 100-300 nm) formed in the stationary phase, and (iv) OMVs with a rough surface formed in the late stationary phase. Exposure of *A. baumannii* to a sub-inhibitory concentration of the antibiotic ceftazidime resulted in filamentous forms that produced large numbers of OMVs. We argue that the different types of membrane vesicles formed during the various stages of the life cycle of *A. baumannii* play distinct roles in infection.

Introduction

Acinetobacter baumannii is a Gram-negative bacterium that can colonize and infect severely ill, hospitalized patients. Multidrug-resistant strains of *A. baumannii* have the propensity to spread among patients and numerous endemic and epidemic episodes caused by this species have been reported [13,32]. Although colonization is far more common than infection, severe *A. baumannii*-associated infections, including ventilator-associated pneumonia and catheter-related bloodstream infections, do occur [32]. Several virulence attributes are thought to play a role in *A. baumannii* infections, including its ability to adhere to and invade host cells [7,10,11,25], form a biofilm, induce host cell death [6], resist the killing actions of serum [20] and produce siderophores [15].

It has been reported that virulence factors of Gram-negative bacteria can be delivered to host cells by outer membrane vesicles (OMVs) [18,22,23]. OMVs are nanovesicles composed of outer membrane and periplasm components, such as phospholipids, proteins, and lipopolysaccharides (LPS) [1,26]. However, OMVs produced by a clinical *A. baumannii* isolate were found to contain not only proteins from the outer membrane, including the potent cytotoxic outer membrane protein A (OmpA), and the periplasm, but also components from the inner membrane and cytoplasm [23]. OmpA present on OMVs of *A. baumannii* can contribute directly to host cell death [18], indicating that OMVs from *A. baumannii* are an important vehicle to deliver bacterial effector molecules to host cells. Moreover, it has been suggested that *A. baumannii* release OMVs as a mechanism of horizontal gene transfer whereby carbapenem resistance genes can be delivered to surrounding *A. baumannii* isolates [34]. It is of note that these studies on the composition and function of OMVs of *A. baumannii* all used extensive purification methods for the isolation of OMVs, including (ultra)-filtration and centrifugation. A disadvantage of this isolation procedure could be the presence of inner membrane and cytoplasmic proteins in OMVs resulting from random capture by membrane fragments. Thus, these preparations may not represent the naturally occurring OMVs [22]. The aim of the present study was to characterize the formation and morphology of naturally formed membrane vesicles by *A. baumannii* ATCC19606^T during the various stages of its life cycle using cryo electron tomography.

Material and Methods

Bacterial strain and culture conditions

A. baumannii ATCC19606^T was used in the present study. Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at -80°C. For experiments, inocula from fresh overnight cultures on sheep blood agar plates were grown overnight in Luria Bertani (LB) medium at 37°C under shaking. One hundred microliters of the overnight culture were added to 15 ml of prewarmed LB medium in an Erlenmeyer flask and incubated at 37°C under vigorous shaking. Where indicated, bacteria were cultured for 2.5 h at 37°C under vigorous shaking in the presence of 4 mg/L ceftazidime (Sigma-Aldrich, Zwijndrecht, The Netherlands), i.e. below the MIC level of 16 mg/L of *A. baumannii* ATCC19606^T [8].

Isolation of vesicles

Bacterial suspensions were cultured for 1, 2.5, 6, 20, and 48 h in LB medium at 37°C. Thereafter the bacteria were centrifuged for 10 min at 3,000 g and the supernatants were centrifuged for 1 h at 17,000 g to remove remaining cells and debris. Next, the pellets were resuspended in 1 to 2 ml phosphate buffered saline (PBS; pH 7.4) and these preparations were used for analysis by cryo electron microscopy.

Cryo electron microscopy

Cryo sample preparation

A few microliters of vesicle preparation were applied to glow-discharged lacey carbon EM grids. For cryo electron tomography, 5 or 10 nm protein A - gold particles were added as fiducial markers to the sample. Excess medium was automatically blotted onto Whatman no. 4 filter paper for 1 to 2 sec in a controlled environment operated at room temperature and 100% humidity. Subsequently, the specimen was vitrified by plunging into liquid ethane in a Vitrobot Mark IV (FEI Company, Eindhoven, The Netherlands). Samples were stored in liquid nitrogen until use. Grids were mounted in a Gatan 626 cryo holder (Gatan, Pleasanton, Germany) for cryo electron microscopy imaging.

Cryo electron microscopy

Cryo electron microscopy (cryo EM) was performed on several microscopes. For large scale automated data collection we used custom written software (unpublished) that was able to automatically scan and image large areas. These images were recorded on a Tecnai 12 electron microscope with a LaB_6 source and operated at 120 keV using a 4k x 4k Eagle camera (FEI Company). Cryo electron tomography (cryo ET) was performed on a Tecnai 20

FEG operated at 200 keV and a Tecnai G2 Polara operated at 300 keV (FEI Company). Images were recorded using Explore 3D software on a 2k x 2k camera mounted behind a GIF energy filter (Gatan) operated at a slit width of 20 eV. Cryo ETs of membrane vesicles were recorded with 2° tilt steps between -70° to +70° at a defocus of -5 micron, while tomograms of whole bacteria were recorded between -60° to +60° at a defocus between -6 and -8 micron and a magnification between 13,500 and 19,000 corresponding with a pixel size of 1.02 resp. 0.69 nm.

Image analysis and visualization

Tomographic tilt series were processed using IMOD version 3.9 [21]. Projection images were roughly aligned by cross-correlation and fine alignment was done using fiducial markers. The tomograms were reconstructed using weighted back-projection. Cryo electron tomograms were denoised using non-linear anisotropic diffusion. The number of OMVs and their size were measured using ImageJ [33]. Image segmentation and 3D surface rendering was performed using AMIRA version 5.2 (Visage Imaging, Berlin, Germany).

Results

Acinetobacter morphology

We investigated the general morphology of logarithmic phase *A. baumannii* ATCC19606^T using cryo EM/ET (Figure 1). The bacteria did not form aggregates and readily attached to the carbon surface of the electron microscopy grid (Figure 1A). The size of (visibly classified) non-dividing bacteria was 1.54 ± 0.07 micron in length and 0.77 ± 0.04 micron in width (n=9). The bacterial outer membrane was smooth (Figure 1B) with occasional small ripples, predominantly at the distal ends. The distance between the outer and inner membrane layers was 33.4 ± 1.48 nm and the peptidoglycan layer measured about 7 ± 0.7 nm in width and was centered between the inner and outer membrane (Figure 1C, D).

The bacterial cell surface was covered by a ~150 nm thick layer with very low contrast and a radial appearance (data not shown). Also long filaments, bacterial fimbriae or pili, were extending from the surface of the bacteria as was observed earlier by others (11). The bacteria regularly contained 50-500 nm dark inclusions (Figure 1B) possibly containing phosphor, magnesium and potassium [3,16].

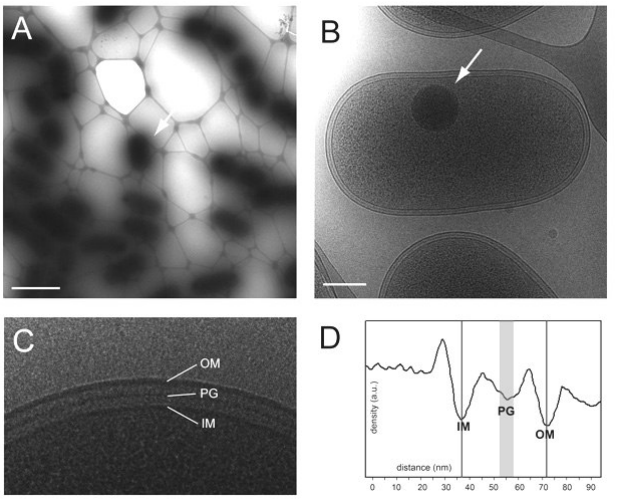

Figure 1. Cryo electron microscopic overview of *A. baumannii* ATCC19606^T morphology and membrane structure. A*, A. baumannii* ATCC19606^T (white arrow) on a lacey carbon support. B, Typical morphology of a single bacterium. In many cells a dark inclusion is present (white arrow). C, Detailed image of the bacterial membrane, showing the inner membrane (IM), the peptidoglycan layer (PG) and the outer membrane (OM). D, Density profile of the bacterial membrane showing the inner membrane, peptidoglycan and outer membrane. Scale bars are 2 µm (A) and 200 nm (B).

Formation and structure of membrane vesicles during logarithmic phase

In order to investigate whether the morphology and formation of membrane vesicles depends on the stage of the bacterial life cycle we imaged the vesicles from *A. baumannii* ATCC19606^T cultured for 1 and 2.5 h (early and mid-logarithmic phase), 6 h (late logarithmic phase), and 20 and 48 h (early and late stationary phase, respectively).

Cryo ET recordings from 1 and 2.5 h cultured *A. baumannii* revealed the presence of ~30 nm vesicles budding off from the outer membrane (Figure 2B - G). These outer membrane vesicles (OMVs) were rarely observed possibly due to their small size and/or their limited occurrence. The formation of OMVs mainly occurred at the bacterial distal ends. At these sites we noted that the peptidoglycan layer was thickened and less dense, and the distance between inner and outer membrane was increased (Figure 2 A-F). In addition, large OMVs - typically 200-500 nm in diameter - were formed predominantly at sites of bacterial cell division (Figure 3A, B). Cryo ET on large OMVs showed that these vesicles lacked internal structures and displayed a smooth appearance (Figure 3C, D). These OMVs lacked fimbriae and the previously noted 150 nm thick low contrast layer that is present on the whole bacterium. Cryo electron microscopic images of OMV

formation indicated that these OMVs are connected to septa via a small outer membrane tubal structure (Figure 3B, E).

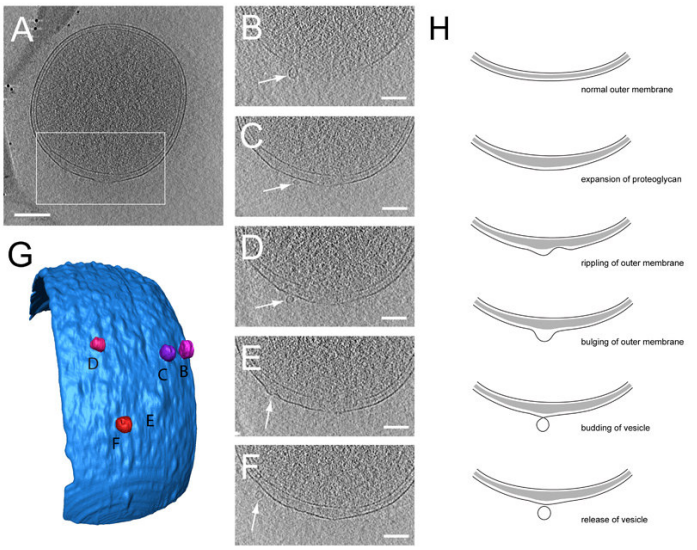

Figure 2. Formation of small outer membrane vesicles during logarithmic growth phase. A, Cryo electron tomographic slice of *A. baumannii*. B-F, Tomographic slices through the tomogram from the area that is outlined in (A) with white arrows indicating budding of outer membrane vesicles (OMVs). G, Surface representation of part of the bacterium that is outlined in (A) with colored in blue the bacterial outer membrane and in red to purple the formed OMVs. H, Schematic representation of the formation of small OMVs. Scale bars are 200 nm (A) and 100 nm (B to F).

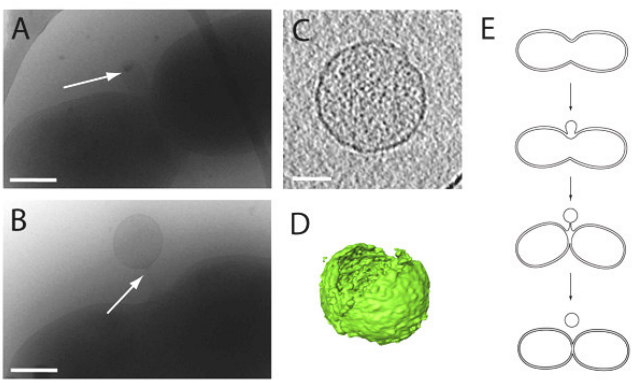

Figure 3. Formation of large outer membrane vesicles during logarithmic growth phase. A, Outer membrane vesicles (OMVs; white arrow) are often formed at or in the vicinity of bacterial septa during cell division. B, Sometimes OMVs are attached to the cell wall of dividing bacteria via a long tube (white arrow). C, Slice from a cryo electron tomogram through a large OMV reveal a smooth surface. D, 3D reconstruction of the tomogram in C. E, Schematic representation of the various stages of formation of large OMV at septa. Scale bars are 300 nm (A and B) and 50 nm (C).

Formation and structure of membrane vesicles during stationary phase

While in early (<6 h) bacterial cultures the number of vesicles was low, it dramatically increased during stationary phase (>6 h) cultures (Figure 4). The number of membrane vesicles did not linearly increase with the number of viable bacteria in the culture medium, suggesting that membrane vesicles are primarily formed from stationary bacteria and/or dying bacteria.

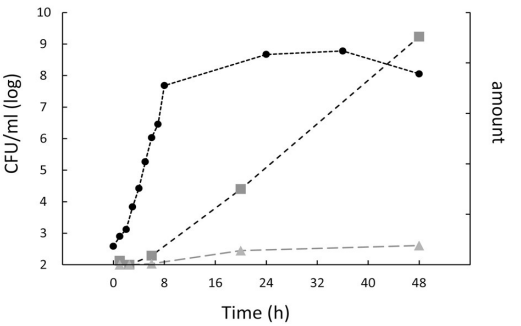

Figure 4. *A. baumannii* growth curve and membrane vesicle formation. The growth curve of *A. baumannii* ATCC19606^T is plotted (round dots and black line) and the number of outer membrane vesicles (squares and dark grey striped line) and inner and outer membrane vesicles (triangles and light grey striped line) are plotted in time.

Also from cryo EM it appeared that the majority of membrane vesicles was formed by cellular degradation. While logarithmic phase *A. baumannii* exhibited a very regular shape, stationary bacteria often displayed anomalous shapes, such as bulges of the outer membrane (Figure 5A - C). It seemed that these deviating bacterial shapes are intermediates of bacterial fragmentation (Figure 5C, D) possibly associated with the process of bacterial cell death. Vesicles pinched off from dying bacteria, resulted in the formation of vesicles containing both inner and outer membranes (IOMV; Figure 5C, E - G, left panel), as demonstrated by tomographic reconstruction (Figure 5E, F). Probably IOMVs contain all components that are present in the periplasmic space and the cytoplasm (e.g. DNA). Almost all IOMVs contained a 150 nm low dense layer and fimbriae on their surface. These were occasionally observed in OMVs in stationary phase bacteria.

It appeared that in several IOMVs the inner membrane was dissociated or totally detached from the peptidoglycan layer, while this peptidoglycan layer remained associated with the outer membrane (compare Figure 5E and Figure 1C). Additionally, we observed intermediate structures with evidence of gradual breakdown of the inner membrane (Figure 5G) and secondary vesicle formation, i.e. membrane vesicle formation from IOMVs (Figure 5G, right panel). This breakdown of IOMVs and the formation of

membrane vesicles from IOMVs could explain the decrease in the relative numbers of IOMVs compared to the numbers of OMVs in later growth stages (Figure 4). The size of the membrane vesicles increased with the development from early logarithmic phase towards late stationary phase; OMVs being 72.3 ± 68.4 nm, while IOMVs were about 2 to 3 times larger (189 ± 115 nm).

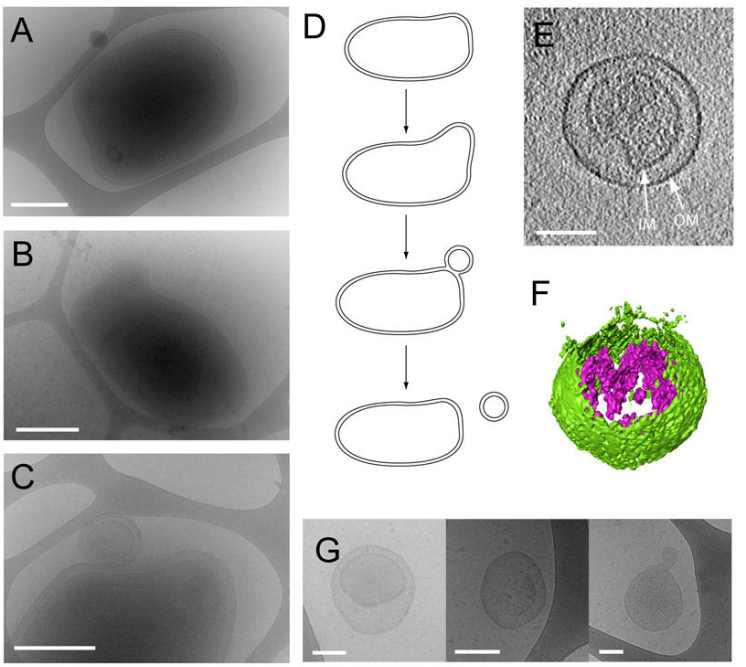

Figure 5. Formation of inner and outer membrane vesicles during late stationary phase. A, B, Bacteria deform and exhibit bulges that result in the (C) formation of membrane vesicles comprising both inner and outer membranes and peptidoglycan. D, Schematic representation of the stages of the formation of inner and outer membrane vesicles (IOMVs). E, Slice from a tomogram of an IOMV, with arrows showing the inner membrane (IM) and outer membrane (OM). F, 3D reconstruction of the tomogram in E, with in green the outer membrane and in purple the inner membrane. G, Once the IOMVs have been released from the bacteria they can have different shapes and the peptidoglycan and the inner membrane seem to degrade. Additionally, it appeared that MVs can arise from larger MVs. Scale bars are 300 nm (A, B and C) and 100 nm (E and G).

In *A. baumannii* cultures occasionally bacterial remnants were observed. In the most extreme case the bacterial remnants were transformed into a branched arrangement from which at the end OMVs were released (Figure 6A, B). These OMVs (Figure 6C - E) were rather uniform in size measuring roughly 75 nm. Cryo ET showed that the surface of

these vesicles had a rough appearance, being covered with small and large densities, most probable representing membrane (-associated) proteins (Figure 6E). On almost all these OMVs a large (10 nm) density was present (Figure 6D, E) that, judged by its shape and size, could be hemolysin (37). Large numbers of these rough OMVs were observed in the vicinity of bacterial remnants and given the surface area of an *A. baumannii* and the average radius of the OMVs, between 100 and 400 vesicles could be derived from a single bacterium.

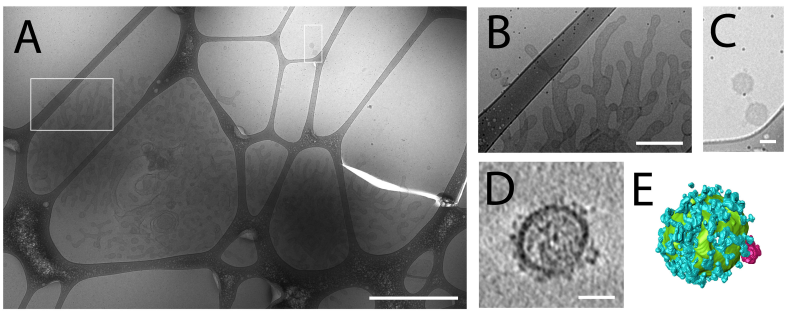

Figure 6. Formation of outer membrane vesicles from late stationary phase bacteria. A, outer membrane vesicles (OMVs) were formed form bacteria in their late stationary phase. B, From branching membrane structures OMVs are released (area from large white box in A). C, These OMVs are round and have a rough surface (area from large white box in A). D, Cryo electron tomography showed that these OMVs have many small densities on their surface and regularly contain a large cylindrical density. E, 3D reconstruction of the tomogram from D more clearly shows the rough surface and the densities that are suggestive for proteins (blue), the lipid membrane (green) and the large cylindrical protein complex (purple). Scale bars are 1 µm (A), 200 nm (B) and 50 nm (C) and 25 nm (D).

Effect of ceftazidime on vesicle formation

A sub-inhibitory concentration of the cephalosporin ceftazidime, which interferes with peptidoglycan synthesis, was added to *A. baumannii* cultures to investigate the effects of this antibiotic on the formation of OMVs. As expected in 2.5 h exposed cultures many filaments had been formed; in most extreme cases reaching lengths up to several tens of microns (Figure 7A). Membrane ruffling was observed along the whole outer membrane of ceftazidime-exposed bacteria instead of being localized solely to the distal ends of control bacteria. Additionally, deformations of the peptidoglycan layer and the outer membrane of antibiotic-exposed bacteria were observed frequently. In the presence of ceftazidime, membrane vesicle formation was more abundant than in the absence of the antibiotic. Especially at sites of potential septum formation (Figure 7B, C) and at distal ends (Figure 7D - F) membrane vesicle formation was more frequent than in control *A.*

baumannii. Cryo ET showed that these membrane vesicles often contained peptidoglycan at the inside and fimbriae and the low contrast layer on the outer surface (Figure 3G, H).

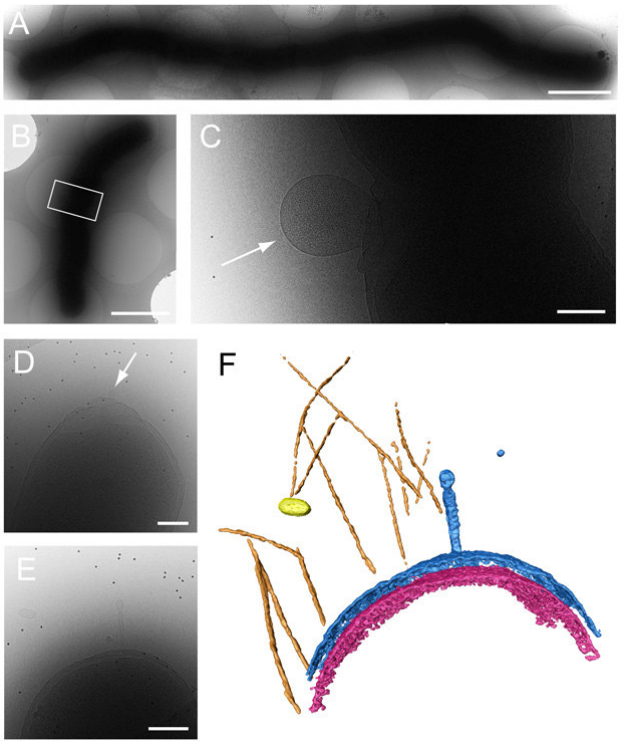

Figure 7. Formation of outer membrane vesicles by ceftazidime-treated bacteria. A, Ceftazidime inhibits bacterial division resulting in the formation of long bacterial structures. B, C, The formation of outer membrane vesicles (OMVs) was observed at potential sites of cell division with membrane roughing and peptidoglycan expansion (the white box in B depicts the position of C). D, At the bacterial distal ends, broadening of the peptidoglycan layer and formation of OMVs was regularly observed (white arrow). E, A large extension of the outer membrane. F, 3D reconstruction of the area in D shows OMVs (yellow) with attached fimbriae (orange), the formation of OMVs by bulging of the outer membrane (blue) and the inner bacterial membrane (purple). Scale bars are 2 µm (A and B), 200 nm (C to E).

Discussion

The main finding of the present study is that *A. baumannii* ATCC19606^T forms morphologically diverse types of membrane vesicles during different stages of its life cycle. In exponentially growing bacterial cultures outer membrane vesicles (OMVs) were

formed mainly at distal ends of the bacteria and at septa of dividing cells. Stationary phase and dying bacteria released large numbers of vesicles, i.e. rough OMVs and inner and outer membrane vesicles (IOMVs), the latter were formed by partial fragmentation and budding off the cells. The timing of IOMV formation suggests that they are mainly derived from dying bacteria. These OMVs and IOMVs were abundant and it appeared that their surfaces were covered with low contrast material with a radial appearance, fimbriae and protein complexes. This is in accordance with the mass spectrometry measurements on purified membrane vesicles from *A. baumannii* [24]. It is conceivable that the biogenesis of membrane vesicle formation by *A. baumannii* in the present in vitro cultures may also occur in vivo. For example, Jin et al demonstrated that *A. baumannii* ATCC19606^T secreted membrane vesicles during pneumonia in a mouse model of infection [18]. Others reported that *A. baumannii* present in alveolar macrophages in a patient with fatal *A. baumannii* pneumonia secreted multiple pleomorphic vesicles [28], suggesting the possibility of secretion of different types of vesicles during infection.

What are the functions of the various types of membrane vesicles in *A. baumannii* infection? It has been reported that pathogenic Gram-negative bacteria, including *A. baumannii*, deliver toxins and other virulence attributes to host cells via OMVs [2,17-19]. So far, proteomic studies on OMVs from *A. baumannii* including the ATCC19606^T strain have indicated that inner membrane and cytosolic proteins as well as some virulence factors were present in these vesicles [19,23]. In addition, IOMVs of *A. baumannii* contain cytoplasmic material, including genetic material, and therefore they could be involved in gene transfer among bacteria. It should be noted that knowledge of the transfer of genetic material among clinical *A. baumannii* strains is still very limited. Together, further studies are clearly needed to identify the virulence attributes present in the various membrane vesicles formed by *A. baumannii* during its life cycle.

Other important findings pertain to the main mechanisms underlying the biogenesis of membrane vesicles in *A. baumannii*. Several genetic, biochemical, proteomic and microscopic observations have indicated that disruption of peptidoglycan synthesis genes and the removal of proteins that interconnect peptidoglycan-outer membrane interactions can lead to increased formation of OMVs [12,27]. Our cryo electron tomographic data showed that OMVs were formed primarily at sites where reorganization of peptidoglycan occurred, i.e. the peptidoglycan layer increased in thickness and was deformed, thus supporting the current models for OMV formation.

We found that sub-inhibitory concentrations of ceftazidime, which interferes with peptidoglycan synthesis by inhibiting the penicillin-binding-proteins (PBPs) in the cytoplasmic membrane of Gram-negative bacteria [9], resulted in filament formation, as described before [4]. These strands of non-dividing bacteria contain enhanced quantities

of LPS conferring the risk of delayed, higher release of LPS during treatment of infections [14]. Moreover, these sub-inhibitory concentrations of ceftazidime enhanced OMV production at distal and septal sites. 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.

In this study we investigated both the formation and three-dimensional structure of naturally occurring membrane vesicles from *A. baumannii* using cryo electron tomography. To ensure optimal preservation of membrane vesicles from *A. baumannii* ATCC19606^T, we isolated the membrane vesicles from bacterial cultures by centrifugation steps only. This lenient isolation procedure limits rupture of cells and vesicles, stripping proteins from the surface and induction of vesicle fusion [22] resulting from harsh purification methods, that are regularly used in other studies. Furthermore, we employed cryo fixation of the samples using vitrification to preserve the membrane vesicles with high integrity. Cryo electron tomography has been used since this method is able to resolve cellular structures with high accuracy and resolution in three dimensions. In earlier investigations on the structure and formation of membrane vesicles either negative staining of purified MVs was employed or fixation, dehydration, staining and sectioning techniques were used [18,29-31,35]. In line with the above considerations, cryo electron tomography is increasingly used to study bacteria [5,36,38].

Our findings show that several types of membrane vesicles are formed by *A. baumannii* ATCC19606^T during the different stages of the bacterial life cycle and we cannot exclude that these different types exert specific functions and have clinical significance.

Acknowledgements

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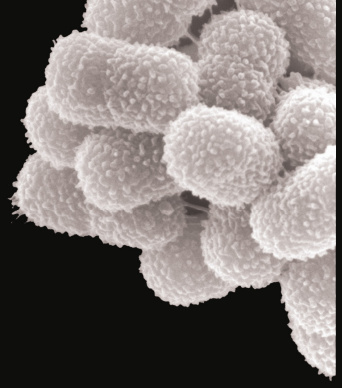

Chapter 7

Three-dimensional human skin equivalent as a tool to study *Acinetobacter baumannii* colonization

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Abstract

Acinetobacter baumannii can colonize body surfaces of hospitalized patients. From these sites, invasion into the host and spread to other patients and the hospital environment may occur. Eradication of the organism from the patient's skin is an important infection control strategy during epidemic and endemic episodes. In this study, a three-dimensional, air-exposed human epidermal skin equivalent was exploited to study *Acinetobacter* skin colonization. We characterized the adherence of *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T to and biofilm formation on the skin equivalent and the responses to these bacteria. Furthermore, we assessed the ability of the disinfectant chlorhexidine to decolonize the skin equivalents. Results revealed that both strains replicated on the stratum corneum up to 72 h, but did not invade the epidermis. *A. baumannii*, in contrast to *A. junii*, formed large biofilms on the stratum corneum. Bacterial colonization did not affect keratinocyte activation, proliferation or differentiation nor did it induce a strong inflammatory response. Disinfection with chlorhexidine solution resulted in complete eradication of *A. baumannii* from the skin without detrimental effects. This 3D model is a promising tool to study skin colonization and to evaluate the effects of novel disinfectant and antimicrobial strategies.

Introduction

Multidrug resistant (MDR) strains of *Acinetobacter baumannii* are notorious for their association with outbreaks of colonization and infection worldwide [6,23]. During such outbreaks, *A. baumannii* can colonize body surfaces of severely ill patients from which the organisms may invade the patient causing infection and/or spread to other patients and their environment. Thus, the skin is thought to constitute an important reservoir for *A. baumannii* during outbreaks and endemic episodes [1,17]. Insight into *Acinetobacter* skin colonization and the microbial ecology of the skin may result in novel strategies to prevent or interfere with skin colonization and thus contribute to eradication of the organisms from a ward.

Adherence and biofilm formation on plastic and adherence to human cells are widely used systems to study interactions of bacteria with abiotic and biotic surfaces. However, these systems may not adequately reflect the association of bacteria with the human skin, a process that takes place under relatively dry conditions [20]. Moreover, adherence and subsequent replication is strongly influenced by environmental conditions [19], including the physico-chemical barrier properties of the skin surface and its nutrient availability. Once bacteria have adhered to the skin, they may invade the epidermis and trigger an inflammatory response. To our knowledge little is known about the possible response of human skin cells to *Acinetobacter*.

Tissue-engineered, air-exposed human skin models are 3D systems that mimic the native skin to a high degree [10,11]. Such epidermal skin equivalents are generated by culturing primary keratinocytes at the air–liquid interface on cell-free matrices (e.g., inert filters or de-epidermized dermis). The keratinocytes will proliferate, migrate and differentiate during epidermal development resulting in skin equivalents that contain all layers of the native epidermis [11]. The skin equivalents also have barrier properties that show high similarities with the human skin [21]. These systems are interesting models for studying pathogen-skin interactions.

In the present study, we exploited a 3D human epidermal skin equivalent to study the adherence of an *A. baumannii* and an *A. junii* strain to the skin, subsequent biofilm formation and the skin's response to these bacteria. Furthermore, we explored the usefulness of this model to investigate the effects of a disinfectant on the bacteria and the human skin.

Materials and methods

Generation of epidermal skin equivalents

Human keratinocytes were isolated from fresh plastic surgery surplus skin as previously described [10]. Briefly, the epidermis and dermis were enzymatically and mechanically separated and each layer subsequently digested to obtain single cell suspensions. Keratinocytes were cultured in Dermalife medium (Lifeline cell technology) supplemented with penicillin (10000 U) and streptomycin (10 mg/ml). Epidermal skin models were generated as described [11,21]. In short, approximately $2x10^5$ keratinocytes from a secondary culture were seeded onto a filter insert (12 mm in diameter, Costar, Corning) in 12-wells plates in Dermalife medium. Three days after seeding, cells were put air-exposed by aspirating the apical medium. The basal medium was replaced with CnT-02-3D medium (CellTec) supplemented with 2.4×10^{-2} µM bovine serum albumin, 25 µM palmitic acid, 15 µM linoleic acid and 7 µM arachidonic acid. Prior to bacterial inoculation, the medium was replaced by keratinocyte medium without penicillin and streptomycin. Experiments were performed using 7 days air-exposed cultures.

Preparation of bacterial inoculum

A. baumannii type strain ATCC19606^T and *A. junii* type strain ATCC17908^T (=RUH2228^T) were used. Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at -80°C. Inocula from frozen cultures were grown overnight at 37°C on sheep blood agar plates (BioMerieux). Bacteria were cultured for 2.5 h at 37°C in Luria Bertani (LB) medium [10 g of Bactotryptone, 5 g of Yeast extract (both from BD, Sparks) and 5 g of sodium chloride (Merck) in 1 l of distilled water] under vigorous shaking. This suspension was diluted in phosphate buffered saline (PBS; pH 7.4) to a concentration of approximately $3x10^5$ colony forming units (CFU)/ml as calculated from the absorbance of the suspension at 600 nm, and verified afterwards using standard vital counts.

Colonization of epidermal skin equivalents

Skin equivalents were incubated with 300 µl of the bacterial suspension at 37°C / 7.3% CO_2. After 1 h, the bacterial suspension was aspirated to remove non-adherent bacteria. At different intervals after inoculation, the number of viable detachable and adherent bacteria was assessed microbiologically. Briefly, 600 µl of PBS were applied to the skin and the detachable bacteria were collected, serially diluted and plated onto diagnostic sensitivity test (DST) agar plates to determine the number of CFU. To assess the number of adherent bacteria, two biopsies (each 4 mm in diameter) were taken from the skin, homogenized in PBS using a glass Potter-Elvehjem tissue homogenizer and the

homogenates were subsequently serially diluted. The lower limit of detection of detachable and adherent bacteria was 12 and 115 CFU/skin equivalent, respectively. The number of adherent bacteria per skin equivalent (113.04 mm^2) was calculated by multiplying the number of adherent bacteria in two biopsies (25.12 mm^2) by 4.5. The total number of bacteria per skin equivalent was calculated by adding up the number of detachable bacteria per skin equivalent and the number of adherent bacteria per skin equivalent.

Microscopic analysis of bacterial replication on skin equivalents

To visualize bacterial colonization of the skin equivalents, one biopsy of each skin equivalent was fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Next, paraffin blocks were cut into 5 µm sections, deparaffinized and rehydrated and then stained with Alcian-blue in combination with Periodic acid-Schiff (PAS, both from Merck) to detect polysaccharides.

Immunohistochemical analysis of keratinocyte response

For analysis of the effects of colonization of the skin equivalents, the levels of keratin (K) 16, a marker for keratinocyte activation or stress; Ki67, a marker for keratinocyte proliferation; and K10, a marker for early keratinocyte differentiation were determined by immunohistochemical analysis. In short, standard antigen retrieval of deparaffinised and rehydrated paraffin sections was performed by immersing sections in 0.01 M citrate buffer (pH 6.0) for 30 min at 90°C followed by slow cooling down to room temperature for at least three h prior to staining of the sections. Sections were incubated overnight at 4°C with the primary mouse antibodies directed against human K16 (AbD serotec, clone LL025, 5x diluted), Ki67 (DAKO, clone MIB-1, 75x diluted), and K10 (Abcam, clone DE-k10, 50x diluted). Thereafter, sections were incubated for 60 min with secondary biotinylated goat-anti-mouse antibodies (DAKO) and subsequently 30 min with streptavidin-biotinylated horseradish peroxidase (DAKO). Chromogen 3-amino-9-ethyl-carbozole (AEC) solution was used as substrate solution. Sections were washed three times with PBS between subsequent incubations and finally counterstained with haematoxylin.

To determine the proliferation index, the number of Ki67 positive nuclei from the total number of basal cells was used. A minimum of 100 basal cells was counted in sections of three different samples at a magnification of 200x.

Determination of cytokine and chemokine levels

The levels of IL-1α, IL-1β, IL-6, IL-8, IL-10, and TNFα in culture media were determined by ELISA (all from Biosource, Invitrogen) according to manufacturer's instructions. The lower

limit of detection was 1 pg/ml for IL-1α and IL-1β, 15 pg/ml for IL-6, 7 pg/ml for IL-8 and 25 pg/ml for IL-10 and TNFα.

Determination of gene expression levels

Total RNA was extracted from homogenized skin biopsies using the RNeasy Mini kit (Qiagen), followed by a treatment with DNase I (Qiagen). cDNA synthesis was performed on 300 ng of total RNA using the iScript cDNA synthesis kit (Bio-RAD) following manufacturer's instructions. For each sample, a control for genomic DNA contamination was included by adding sterile water instead of reverse transcriptase. Real-time quantitative PCR was performed in an ICycler IQ (Bio-RAD) in a final volume of 25 µl comprising 1x IQ SYBR Green Supermix (Bio-RAD), 10 pmol of each primer (Table 1) and 5 µl of 10x diluted cDNA. PCR conditions consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 56.5°C (for Ki67), 57.8°C (for LL-37), 60.8°C (for GAPDH and IL-1β), 61.7°C (for TNFα) or 62°C (for β2M, IL-1α, IL-6, IL-8, hBD-2, hBD-3, K16 and K10) for 15 s, and elongation at 72°C for 20 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-2 microglobulin (β2M) were used for standardization. Experiments were performed in duplicate. Data were acquired and analyzed using ICycler IQ Optical System software (Bio-RAD) with automatic adjustment of the baseline and threshold parameters.

Table 1. Primer sequences

	Forward	Reverse
β2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
GAPDH	AAGGTCGGAGTCAACGGATTT	ACCAGAGTTAAAAGCAGCCCTG
IL-1α	CGCCAATGACTCAGAGGAAGA	AGGGCGTCATTCAGGATGAA
IL-1β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACTGAC
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-8	GCCAGGAAGAAACCACCGGAAGG	GGCTGCCAAGAGAGCCACGG
TNFα	CCTGTGAGGAGGACGAACAT	GGTTGAGGGTGTCTGAAGGA
hBD-2	TGATGCCTCTTCCAGGTGTTT	GGATGACATATGGCTCCACTCTTA
hBD-3	TTATTGCAGAGTCAGAGGCGG	CGAGCACTTGCCGATCTGTT
LL-37	ATTTCTCAGAGCCCAGAAGC	CGGAATCTTGTACCCAGGAC
K16	GAGATGCGTGACCAGTACGA	TTGTTCAGCTCCTCGGTCTT
K10	AGCATGGCAACTCACATCAG	TGTCGATCTGAAGCAGGATG
Ki67	AATTCAGACTCCATGTGCCTGAG	CTTGACACACACATTGTCCTCAGC

Decontamination of colonized skin equivalents

Two days after inoculation with *A. baumannii* ATCC19606^T, 0.5% chlorhexidine solution in 70% ethanol or as a control PBS was applied to the skin equivalents using a sterile cotton swab. The numbers of detachable and adherent bacteria were assessed as described above 24 h after application of the disinfectant.

Statistical analysis

Results are expressed as mean ± standard errors of the mean unless stated otherwise. Data were analyzed for statistical significance using the Wilcoxon rank sum test (SPSS 17.0). P values ≤0.05 were considered significant.

Results

Replication of *Acinetobacter* on skin equivalents

To compare the persistence of *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T on the skin, human epidermal skin equivalents were inoculated with approximately 1x10^5 CFU suspended in 300 µl of PBS. The suspensions were aspirated from the skin equivalents after 1 h to allow air-exposure again. The total number of bacteria on the skin equivalent increased during the first 12 h from 7x10^3±6x10^3 to 9x10^7±3x10^6 CFU for the *A. baumannii* strain and from 2x10^4±1x10^4 to 1x10^8±1x10^7 CFU for the *A. junii* strain and thereafter remained stable for the duration of the experiment (Figure 1).

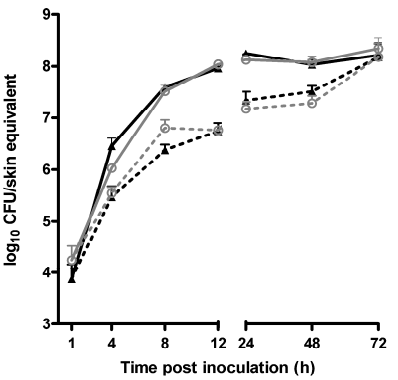

Figure 1. *A. baumannii* and *A. junii* replication on skin equivalents. Human epidermal skin equivalents were inoculated with approximately 1x10^5 CFU of *A. baumannii* ATCC19606^T (triangles) or *A. junii* RUH2228^T (circles). After 1 h, the non-adhered bacteria were removed. At different intervals thereafter, the numbers of detachable and adherent bacteria were determined microbiologically. Results are expressed as mean number of adherent (dotted lines) and total (solid lines) colony forming units (CFU) per skin equivalent ± standard errors of the mean of three independent experiments.

At 48 and 72 h after infection, the number of adhered *A. baumannii* ATCC19606^T was significantly greater than the number of detachable bacteria, indicating that the proportion of bacteria that adhered increased over time (Figure 1). For the *A. junii* strain,

the number of adherent bacteria exceeded that of detachable bacteria at 72 h after infection.

Acinetobacter biofilm formation on skin equivalents

Microscopic analysis revealed that bacteria of both strains persisted on the stratum corneum and did not invade the epidermis (Figure 2). Although there was no significant difference in the number of adherent bacteria between *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T (Figure 1), Alcian blue-PAS staining revealed large biofilm structures by the *A. baumannii* but not by the *A. junii* strain on the stratum corneum at 48 and 72 h (Figure 2).

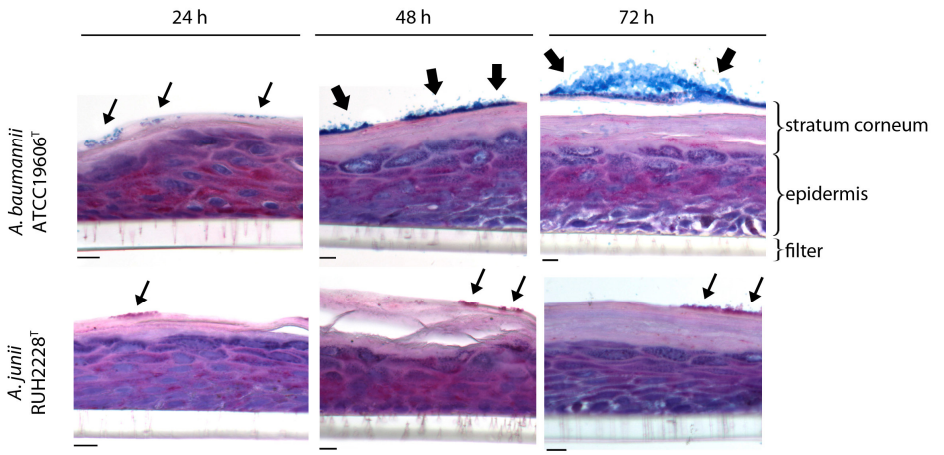

Figure 2. Biofilm formation on skin equivalents. Skin equivalents were exposed to *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T up to 72 h. Thereafter, 5 µm sections of paraffin-embedded skin were stained with Alcian blue PAS and biofilm formation was analysed by light microscopy. Small arrows indicate clusters of bacteria, large arrows indicate bacteria within a biofilm matrix. Scale bars, 20 µm.

Response of skin equivalents to *Acinetobacter*

Subsequently, the effect of bacterial colonization on keratinocyte differentiation, proliferation and cytokine and antimicrobial peptide production was evaluated. Results revealed that exposure of the skin equivalents to *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T did not affect the mRNA expression of Ki67, K10 and K16 in keratinocytes (Figure 3A). In agreement, no difference was seen in protein expression of the activation/stress marker K16 between colonized and PBS-exposed skin equivalents (Figure 3B).

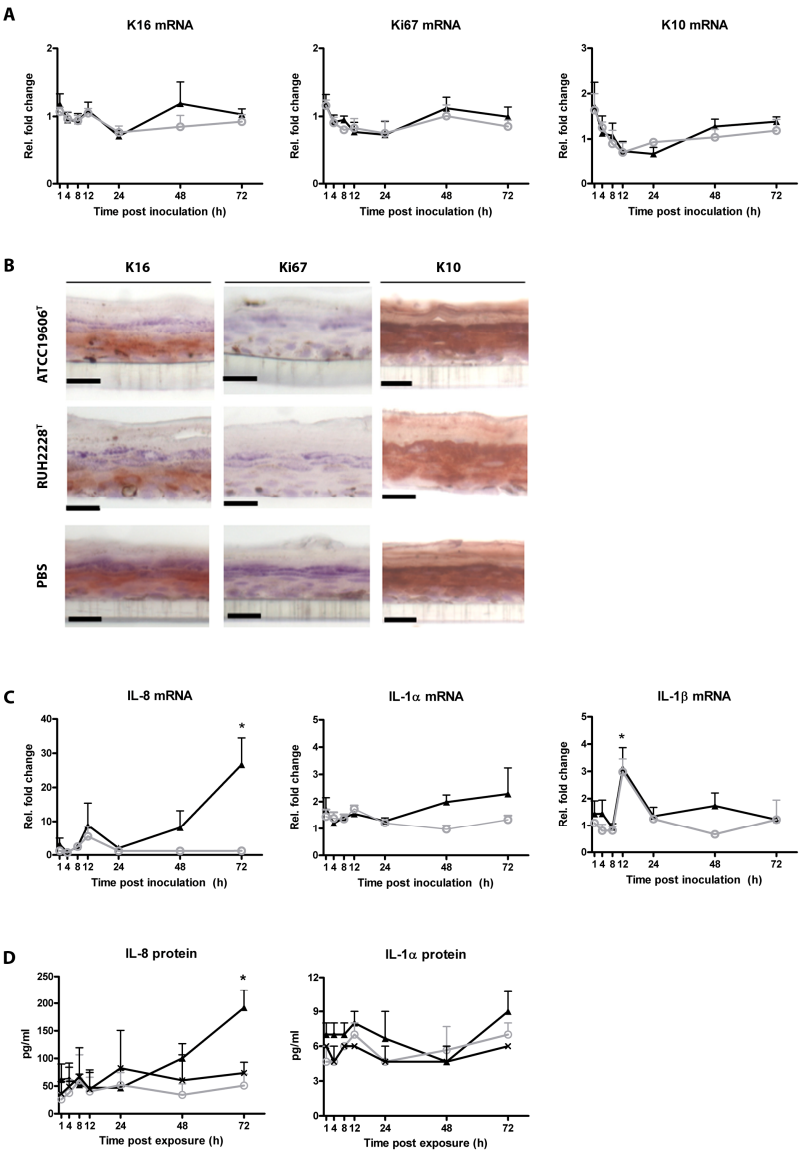

Figure 3. Response of keratinocytes to *Acinetobacter.* Skin equivalents were exposed to *A. baumannii* ATCC19606^T (triangles), *A. junii* RUH2228^T (circles) or as a control PBS (crosses). At various intervals thereafter, mRNA expression (A) and at 48 h thereafter, protein levels (B) of keratinocyte activation/stress marker K16, proliferation marker Ki67 and differentiation marker K10 were determined using qPCR and immunohistochemistry, respectively. Scale bars, 20 µm. In addition, the mRNA expression (C) and protein (D) levels of the chemokine IL-8 and the pro-inflammatory cytokine interleukin (IL)-1α and IL-1β at different intervals were determined using qPCR and ELISA, respectively. Results are expressed as mean fold change in mRNA expression relative to PBS-exposed skin equivalents (A and C) or mean cytokine level in pg/ml (D) ± standard errors of the mean of three independent experiments. *, significantly different from PBS.

Moreover, the expression of K10 in the suprabasal viable cell layers of the exposed and non-exposed skin equivalents confirms that the early differentiation program was not altered by the *Acinetobacter* strains. In addition, the number of proliferating cells in the basal layers was not influenced by the bacteria, i.e. 20±2 versus 18±1 cells positive for Ki67/100 basal cells in *A. baumannii*-colonized skin versus PBS-exposed skin.

Furthermore, we assessed the production of the pro-inflammatory cytokines TNFα, IL-1α, IL-1β, IL-6, and IL-8 and that of the anti-inflammatory cytokine IL-10 by the skin equivalent in response to both *Acinetobacter* strains. During the first 48 h of infection, skin equivalents expressed similar levels of IL-1α and IL-8 after exposure to the two *Acinetobacter* strains as to PBS. However, after 72 h mRNA and protein expression levels of IL-8, but not of IL-1α, were slightly but significantly ($p<0.05$) higher in skin equivalents colonized by the *A. baumannii* strain as compared to those colonized by the *A. junii* strain and/or exposed to PBS (Figure 3C,D). In addition to IL-8 and IL-1α, IL-1β mRNA expression levels were significantly ($p<0.05$) higher in skin equivalents exposed to the *Acinetobacter* strains as compared to PBS-exposed skin equivalents, but only at 12 h of infection (Figure 3C). IL-1β protein was, however, not detectable. Moreover, TNFα, IL-6 and IL-10 mRNA and proteins were not detectable in skin equivalents.

The skin equivalents constitutively expressed the antimicrobial peptides human beta-defensin (hBD)-2 and -3, but no LL-37. Infection of skin equivalents with the *Acinetobacter* strains did not induce enhanced expression of these peptides (data not shown).

Eradication of *A. baumannii* from skin equivalent

Finally, we examined the effect of chlorhexidine on *A. baumannii* ATCC19606^T colonization and on the epidermis. Swapping of skin equivalents colonized by the *A. baumannii* strain for 48 h with 0.5% chlorhexidine solution in 70% ethanol resulted in undetectable levels of *A. baumannii* on the skin (Figure 4A). In addition, this chlorhexidine solution did not affect skin morphology (Figure 4B) and mRNA expression levels of K16, IL-1α and IL-8, but enhanced the expression levels of Ki67 and K10 11-fold and 4-fold, respectively (Figure 4C). This indicates that chlorhexidine enhanced keratinocyte proliferation and differentiation but did not induce epidermal activation/stress or inflammation in the skin (Figure 4C).

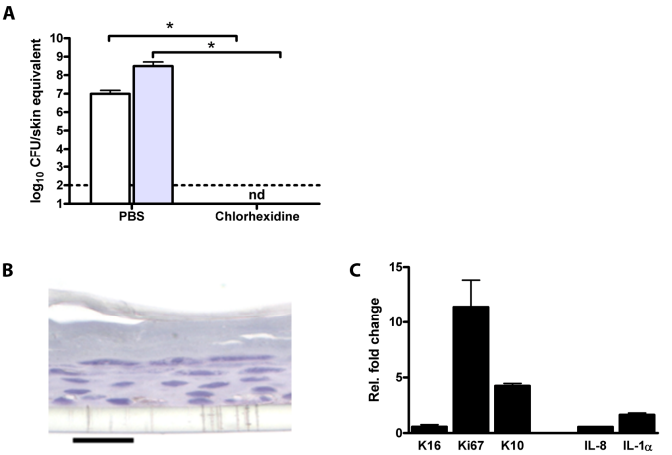

Figure 4. Treatment of infected skin equivalents with chlorhexidine. Skin equivalents were infected with *A. baumannii* ATCC19606^T for 48 h. Thereafter, chlorhexidine or PBS was applied to the skin equivalents. A, Twenty-four hours after treatment, the numbers of detachable and adherent bacteria were determined microbiologically. Results are expressed as mean number of adherent (white bars) and total (gray bars) colony forming units (CFU) per skin equivalent ± standard errors of the mean of three independent experiments. The dotted line represents the lower limit of detection. nd, not detectable; *, significant ($p<0.05$) difference. B, Light micrograph of section of chlorhexidine-treated skin stained with Alcian blue PAS. Scale bar, 20 µm. C, Twenty-four hours after treatment, mRNA expression levels of the keratinocyte activation/stress (K16), proliferation (Ki67) and differentiation (K10) markers as well as the inflammatory mediators IL-8 and IL-1α were determined using qPCR. Results are expressed as mean mRNA expression levels relative to PBS-treated skin ± standard deviations of four measurements.

Discussion

The main conclusion from the present findings is that the 3D human skin equivalent is a promising model to study skin colonization by *Acinetobacter* strains and to evaluate the effects of disinfectants and other antimicrobial agents. This is of importance as *A. baumannii* is able to colonize the skin [2,7,8,17,24], which can be a source for infection and spread to other patients and the environment. Recent papers have emphasized the benefits of skin disinfection as a tool to reduce colonization pressure in a ward [2,12,22]. Our main conclusion is based on the following findings. First, both *A. baumannii* ATCC19606^T and RUH2228^T, which belongs to *A. junii,* a species that occurs on the human skin, adhered to and replicated on the stratum corneum up to 72 h without invading the epidermis. An important difference between these two strains was the ability of the *A. baumannii* strain, but not the *A. junii* strain, to form a large biofilm on the skin equivalents. Secondly, bacterial colonization did not affect keratinocyte activation,

proliferation or differentiation nor did it induce a strong inflammatory response. Thirdly, disinfection with chlorhexidine solution resulted in complete eradication of the *A. baumannii* strain from the skin without detrimental effects.

We have previously shown that both *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T were able to form large biofilms on plastic [5]. On the present human skin model, however, only *A. baumannii* ATCC19606^T formed large biofilms. A possible explanation for the latter observation could be that *A. baumannii*, but not *A. junii*, can utilize the nutrients of the stratum corneum for biofilm formation. In agreement with this suggestion it has been shown that the metabolic versatility of *A. baumannii* is considerably greater than that of *A. junii* [3,18]. The ability of *Acinetobacter* to form biofilms appears to depend on the surface to which the bacteria adhere, as suggested earlier by Gaddy et al [13]. Although more strains should be examined before a definitive conclusion can be drawn, we hypothesize that the ability of *A. baumannii* to form a biofilm on human skin plays an important role in its persistence on the skin.

We have previously shown that human bronchial epithelial cells and cultured human macrophages produce considerable amounts of inflammatory mediators in response to these *Acinetobacter* strains [5]. Using this skin equivalent model, however, *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T induced a poor inflammatory response. This may be explained as follows. In our previous study, the cells were cultured in monolayers and infected with bacteria, enabling direct contact between the bacteria and the cells. However, in the current experiments the bacteria did not invade the epidermis, and thus, did not come in direct contact with live keratinocytes. These results underline the importance of the stratum corneum as a protective barrier against infections, as also shown by Duckney et al, who demonstrated that *Staphylococcus epidermidis* and *Propionibacterium acnes* induced an inflammatory response in a reconstructed human epidermis model only when applied subcutaneously in the culture medium and not when applied topically on the stratum corneum [9]. Moreover, it emphasizes that results from studies into pathogen-host interactions using keratinocyte monolayers (lacking a stratum corneum) will not reflect the in vivo situation.

With the emergence of antibiotic resistance, there has been a re-appraisal of the use of antiseptics for skin disinfection [12,15]. Chlorhexidine is widely used as an antiseptic against many microorganisms, including *A. baumannii* [12]. However, several studies have shown that chlorhexidine may have toxic effects on human fibroblasts [14] and be detrimental to wound healing [14,16], making its use controversial in particular situations. Although the *A. baumannii* type strain used in this study was fully susceptible to treatment with chlorhexidine, a MDR *A. baumannii* isolate that was resistant to chlorhexidine concentrations up to 1% has already been reported [4]. In this light, it is

necessary to develop novel disinfectant strategies to eradicate *A. baumannii* from the colonized skin.

In summary, we have described a novel model for *Acinetobacter* skin colonization using 3D human epidermal skin equivalents. This model will be beneficial in characterizing bacterial growth kinetics and the interactions of different bacterial species on a biotic surface. Moreover, this model may also be advantageous for identifying and evaluating new targets for disinfection and antimicrobial strategies.

Acknowledgements

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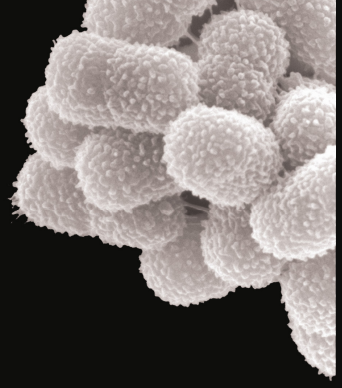

Chapter 8

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*.

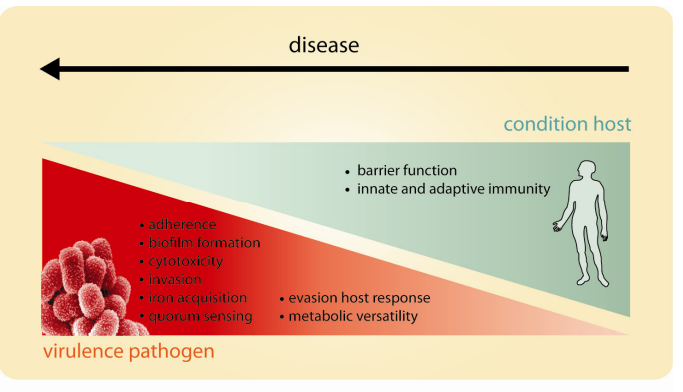

Figure 1. The pathogen-host balance. The outcome of colonization or infection by *A. baumannii* depends both on the virulence of the pathogen (in red) and on the condition of the host (in green).

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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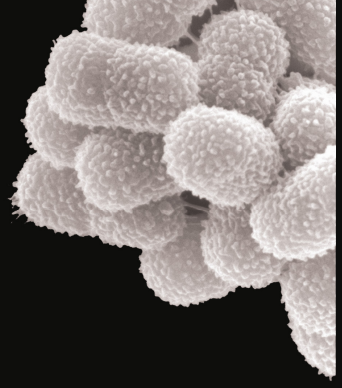

Chapter 9

Dutch summary (Nederlandse samenvatting)

Inleiding

Acinetobacter baumannii is een belangrijke ziekenhuisbacterie die vooral bij ernstig zieke patiënten, zoals patiënten op intensive care afdelingen, aanleiding kan geven tot kolonisatie en infecties. Infecties veroorzaakt door deze bacterie variëren van pneumonie, urineweg-, huid- en wondinfecties tot bacteriëmie met sepsis. Behandeling kan problemen geven omdat stammen dikwijls resistent zijn voor meerdere of zelfs alle bruikbare antibiotica. Het zijn vooral de antibiotica-resistente stammen die zich epidemisch onder patiënten kunnen verspreiden. Er zijn drie groepen van genetisch verwante *A. baumannii* stammen, de zogenaamde Europese klonen I, II en III, die verantwoordelijk zijn voor epidemieën in ziekenhuizen wereldwijd.

Naast *A. baumannii* zijn er twee nauw verwante species, *A. pittii* en *A. nosocomialis*, die relatief vaak in klinische materialen kunnen voorkomen en soms ook antibiotica-resistent zijn. Sommige van de overige *Acinetobacter* species kunnen ook bij de mens voorkomen en infecties geven, maar het beloop van zulke infecties is vaak mild en goed behandelbaar. Of de aanwezigheid van acinetobacters bij een patiënt tot ziekte leidt, wordt bepaald door de balans tussen de weerstand van de gastheer en de virulentie van de bacterie. Er is vooralsnog weinig bekend over de factoren die geassocieerd zijn met de pathogenese van *A. baumannii* infecties. De studies beschreven in dit proefschrift hadden tot doel meer inzicht te krijgen in deze factoren.

Dit proefschrift

Aangezien hechting een cruciale eerste stap is in het kolonisatieproces, hebben wij onderzocht of er verschil bestaat in het vermogen van diverse *Acinetobacter* stammen en species om te hechten aan humaan luchtwegepitheel. De resultaten in **hoofdstuk 2** tonen dat zowel *A. baumannii* stammen als stammen van de klinisch minder relevante species *A. junii* kunnen hechten aan luchtwegepitheel. De mate van hechting varieerde sterk tussen de stammen binnen de twee species. Nadat bacteriën gehecht zijn aan een biotisch of abiotisch oppervlak kunnen zij zich vermenigvuldigen, microkolonies vormen en een beschermende slijmlaag (bestaande uit o.a. exopolysacchariden) produceren. Dit kan leiden tot een gestructureerd netwerk van bacteriën ingebed in een polysaccharidenlaag: een biofilm. Bacteriën in een biofilm zijn resistenter voor factoren van buitenaf, zoals antibiotica en effectorcellen van het immuunsysteem. Het vermogen van een groot aantal *Acinetobacter* stammen en species om biofilm te vormen op een abiotisch oppervlak is vergeleken in **hoofdstuk 2**. Ondanks het verschil in de mate van biofilmvorming tussen

individuele stammen was er geen verschil tussen de klinisch relevante species *A. baumannii*, *A. pittii* en *A. nosocomialis* en de klinisch minder relevante species *A. junii* en *A. calcoaceticus*. Deze studie toont dat hechting aan slijmvlies en biofilmvorming op een abiotisch oppervlak een algemeen kenmerk is van *Acinetobacter* stammen, ongeacht de species.

Tijdens epidemieën vormt de gekoloniseerde huid een belangrijk reservoir van *A. baumannii*. In **hoofdstuk 5** en **7** is een driedimensionaal humaan huidmodel gebruikt om de hechting aan en biofilmvorming op een fysiologisch relevant biotisch oppervlak nader te bestuderen. Zowel stammen van *A. baumannii*, *A. pittii*, *A. nosocomialis* als de klinisch minder relevante *A. junii* konden hechten aan de huidcellen en zich vermenigvuldigen. Opvallend was dat een stam van de klinisch niet-relevante *A. calcoaceticus* niet kon persisteren op het huidmodel. Hoewel al deze stammen een biofilm vormden op plastic, ontwikkelden uitsluitend de drie klinisch relevante species een biofilm op de huidmodellen. Dit suggereert dat biofilmvorming ook afhankelijk is van het oppervlak waaraan de bacteriën gehecht zijn.

Om de verschillen in de mate van hechting en biofilmvorming tussen *Acinetobacter* stammen te kunnen verklaren, onderzochten we mogelijke mechanismen die een rol spelen bij deze kolonisatieprocessen. Pili, haarachtige structuren op het oppervlak van de bacterie, maken hechting van bacteriën aan (a)biotische oppervlakken en aan andere bacteriën mogelijk. In **hoofdstuk 3** is beschreven dat de zogenaamde CsuA/BABCDE-pili, waarvan bekend is dat zij noodzakelijk zijn voor hechting en biofilm vorming van de *A. baumannii* typestam op plastic, geen rol spelen in de hechting van deze stam aan luchtwegepitheel. Ook voor een grotere set *A. baumannii* stammen was er geen relatie tussen de aanwezigheid van pili en de mate van hechting aan epitheel of biofilm vorming op plastic (**hoofdstuk 2**).

Hechting van bacteriën aan de huid of slijmvliezen van de gastheer kan een ontstekingsreactie opwekken, gekenmerkt door de productie van ontstekingsmediatoren zoals chemokines en cytokines en de recrutering van ontstekingcellen naar het geïnfecteerde weefsel. Deze ontstekingscellen spelen in het algemeen een belangrijke rol in het verwijderen van de bacteriën. In **hoofdstuk 2** is de productie vergeleken van specifieke ontstekingsmediatoren door luchtwegepitheelcellen in reactie op *A. baumannii* en *A. junii*. Hoewel stammen van beide species even goed konden hechten aan deze cellen, wekten *A. baumannii* stammen significant minder ontstekingsmediatoren (interleukine (IL)-6) en chemokines (IL-8) op dan *A. junii* stammen. Dit verschil was nog groter in humane macrofagen, die behoren tot de eerste ontstekingscellen waar bacteriën mee in aanraking komen in het weefsel. Aangezien de productie van ontstekingsmediatoren cruciaal wordt geacht voor het verwijderen van de bacteriën,

veronderstelden wij dat *A. baumannii* stammen mogelijk kunnen overleven en persisteren in de luchtwegen van patiënten en ziekte kunnen veroorzaken doordat zij een zwakke ontstekingsreactie opwekken. Deze hypothese hebben wij in **hoofdstuk 4** getoetst met behulp van een muismodel voor pneumonie. Binnen *A. baumannii* was er een duidelijk verschil in het beloop van de luchtweginfectie te zien: *A. baumannii* stammen behorend tot Europese kloon I en II waren zeer virulent, en veroorzaakten hoge morbiditeit en mortaliteit. Een Europese kloon III stam en een sporadische, antibiotica-gevoelige stam vermenigvuldigden zich in dezelfde mate in de longen en verspreidden zich in de bloedbaan van de muis. Echter, deze laatste twee stammen veroorzaakten minder symptomen. De stam behorend tot de laag-virulente species *A. junii* werd snel verwijderd uit de longen en veroorzaakte geen ziekte. Dit zou verklaard kunnen worden door de effecten van een sterke ontstekingsreactie opgewekt door deze stam zoals in vitro het geval was. De typestam van *A. baumannii*, een stam die veelvuldig gebruikt wordt in virulentiestudies, overleefde ook niet in de muis en veroorzaakte geen ziekte. Deze bevindingen benadrukken dat er een groot verschil is in virulentie tussen *A. baumannii* stammen. Met behulp van dit muismodel konden we ook vaststellen dat het aangeboren immuunsysteem van de gastheer verschillend reageert op *A. baumannii* stammen. Zo wekten de minder virulente *A. baumannii* stammen meer specifieke ontstekingsmediatoren (de ontstekingsstimulerende IL-12p40 en IL-23 en het ontstekingsremmende IL-10) op dan de virulente stammen. Verdere studies zullen de rol van deze ontstekingsmediatoren in het beloop van *A. baumannii* infecties moeten ophelderen.

Ook zonder direct contact kunnen bacteriën de gastheercel beïnvloeden, namelijk door middel van blaasjes afgesnoerd van hun buitenmembraan (vesicles), die door de gastheercel kunnen worden opgenomen. Deze vesicles kunnen virulentiefactoren bevatten die langs deze weg in de gastheercel terecht komen. In **hoofdstuk 6** is de productie van membraanvesicles door de typestam van *A. baumannii* onderzocht met behulp van electronen-microscopische technieken. Deze *A. baumannii* stam vormde verschillende typen membraanvesicles, afhankelijk van de groeifase van de bacterie. Vervolgstudies moeten uitwijzen of deze verschillende membraanvesicles virulentiefactoren bevatten en de gastheer beïnvloeden.

Om mogelijke species-specifieke kenmerken te identificeren die het verschil in klinisch gedrag tussen *Acinetobacter* species kunnen verklaren, is een zogenaamde systeemstudie uitgevoerd, waarbij genomische, metabolische en virulentiestudies werden gecombineerd binnen een internationaal samenwerkingsproject (**hoofdstuk 5**). Een divers repertoire aan genen die een rol spelen in het metabolisme werden geïdentificeerd in alle zes geteste *A. baumannii* stammen. Slechts enkele van deze genen waren uniek voor *A.*

baumannii in vergelijking met klinisch minder relevante *Acinetobacter* species. Echter, wanneer het genoom van één *A. baumannii* stam vergeleken werd met dat van een *A. calcoaceticus* stam, werden meerdere unieke genclusters geïdentificeerd, waaronder het *csu* operon (dat codeert voor de hierboven beschreven CsuA/BABCDE-pili) en een gencluster dat een rol speelt bij ijzeropname. Daarnaast lieten de metabolische studies zien dat de *A. baumannii* stam efficiënter stikstofbronnen kon gebruiken en resistenter was voor pH, osmotische en antibiotica stress dan *A. pittii*, *A. nosocomialis* en *A. calcoaceticus* stammen. *A. baumannii* en *A. pittii* konden minder goed fosfaatbronnen gebruiken om te groeien dan *A. nosocomialis* en *A. calcoaceticus*. Juist deze laatstgenoemde species konden niet overleven in een muismodel van een dijbeenspierinfectie. Deze resultaten suggereren dat het klinische succes van *Acinetobacter* species afhankelijk is van genetische verschillen die een functionele impact hebben op metabolische en virulentie-eigenschappen.

Conclusie

De aanwezigheid van meerdere virulentiefactoren in zowel *A. baumannii* als klinisch minder relevante *Acinetobacter* species, toont aan dat het klinische succes van *A. baumannii* niet alleen verklaard kan worden door deze virulentiefactoren. Het vermogen van bepaalde *A. baumannii* stammen om een specifieke gastheerrespons op te wekken in combinatie met hun genetische flexibiliteit, metabolische verscheidenheid en antibiotica-resistentie zijn waarschijnlijk alle belangrijke factoren die geassocieerd zijn met het klinische succes van deze bacterie. Het werk beschreven in dit proefschrift is een stap naar het ophelderen van de factoren die een rol spelen in de pathogenese van *A. baumannii*, wat essentieel is voor de ontwikkeling van nieuwe diagnostische en therapeutische strategieën ten aanzien van infecties door deze bacterie.

Curriculum Vitae

Anna de Breij was born on the 9th of December 1982 in The Hague, the Netherlands. In 2001 she passed her secondary school exam at the Gymnasium Haganum in The Hague and started studying Biology at the University of Leiden. During her studies she performed a first research internship at the department of Medical Microbiology of the Leiden University Medical Center (LUMC) under supervision of dr. E.J. Kuijper, where she developed a toxinotyping method for *Clostridium difficile*. In 2004, she obtained her bachelor's degree in Biology *cum laude*, and started the master Biomedical Sciences at the University of Leiden. Her second research internship at the department of Parasitology of the LUMC under supervision of dr. F. Hartgers focused on the influence of helminth infections on the immune response of children. In October 2005 she started her third internship at the department of Infectious Diseases of the LUMC under supervision of dr. L. Dijkshoorn and dr P.H. Nibbering, addressing the factors that are involved in the pathogenesis of *Acinetobacter baumannii*. After graduating *cum laude* in Biomedical Sciences in 2006, she started the study Medicine at the University of Leiden and obtained her master's degree in Medicine in 2008. Subsequently, she started as a researcher at the department of Infectious Diseases of the LUMC investigating compounds that enhance antibiotic activity to multidrug resistant bacteria in collaboration with the biopharmaceutical company Prosensa under supervision of dr. P. de Visser, dr. P.H. Nibbering and prof. dr. J.T. van Dissel. Furthermore, she continued working on the *Acinetobacter* project under supervision of dr. L. Dijkshoorn, dr. P.H. Nibbering and prof. dr. P.J. van den Broek, which culminated in the work described in this thesis. In October 2011 she pursued her career as postdoctoral researcher in the field of antimicrobial peptides and biofilm infections at the department of Infectious Diseases of the LUMC.

List of Publications

Koning RI, **de Breij A**, Oostergetel GT, Dijkshoorn L, Nibbering PH, Koster AJ. Cryo electron tomographic analysis of membrane vesicle formation by *Acinetobacter baumannii* ATCC19606^{T} at different growth stages. *Submitted*

Peleg AY, **de Breij A**, Adams MD, Cerqueira GM, Mocali S, Galardini M, Nibbering PH, Earl AM, Ward DV, Paterson DL, Seifert H, Dijkshoorn L. The clinical success of *Acinetobacter* species; genetic, metabolic and virulence attributes. *Submitted*

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