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DEDICATION

I want to dedicate this thesis, the hard work of these years, and the achievements to my beloved ones.

To my family.

You gave me freedom to choose, to do, to be. Always supporting me. Allowing me to go far, to chase my dreams. They made me the person I am today.

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How could I survive in these years without you? You were always there, in happy and bad moments. All the time believing in me and supporting me. You taught me to look inside me, how to face problems, and related emotions. There, trying to cheer me up. You are the author, as well, of the person I am today.

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With this thesis ends a chapter of my life. I hope to write more pages together with you in the future.

ABSTRACT

The world-wide spreading of bacteria with multidrug-resistance (MDR) has progressively narrowed the available treatment options. The appearance of dominant MDR *Escherichia coli* and *Klebsiella pneumoniae* clones is particularly concerning. A single *E. coli* clonal lineage, ST131-O25b:H4 already represents 50% of all extended spectrum beta-lactamase producing *E. coli* isolates, and it is expected to be the source of pan-resistant *E. coli* in the future. Here we investigated the *E. coli* ST131-O25b:H4 lineage from several aspects.

First, to better understand the pathogenicity of this family we sequenced, assembled and characterized the genome of our model strain, 81009. Second, we aimed to characterize the role of the complement system in the protection against *E. coli* ST131 infections. The complement system is considered the first line of host defence against Gram-negative bacteria invading the bloodstream and causing bacteraemia or sepsis. However, studying the role of complement in human serum is hampered by the array of other bactericidal factors and by the heterogeneity of pre-existing antibodies in the serum donors. To overcome these problems we developed a new method to reconstitute the alternative and classical pathways from commercially available purified complement factors. Our results proved that the reconstituted complement pathways were active. We also showed that the combination of the alternative and classical pathways with a specific monoclonal antibody (mAb) was effective in killing strain 81009. The components of the reconstituted complement can be individually tailored to the experimental model; thus we expect that it can be used in research areas beyond infectious diseases. Additionally, we studied an isogenic mutant of strain 81009 expressing the colistin resistance gene *mcr-1*. While the expression of *mcr-1* induced resistance to both the bactericidal and lipopolysaccharide (LPS) neutralizing activity of colistin, it did not change the complement-dependent bactericidal and anti-inflammatory activity of an LPS binding mAb. Finally, we explored the prevalence of the ST131 clone among *E. coli* isolates colonizing mechanically ventilated patients in a prospective observational single-center study.

In conclusion, these results show that despite the acquisition of new resistance genes like *mcr-1*, the ST131 clone can be effectively targeted with alternative approaches, like specific bactericidal monoclonal antibodies.

KURZFASSUNG

Die weltweite Ausbreitung multiresistenter (*engl.* multidrug-resistant (MDR)) Bakterien trug progressiv zur begrenzten Verfügbarkeit von Therapeutika bei. Das Auftreten dominanter MDR *Escherichia coli* und *Klebsiella pneumoniae* Klone ist besonders besorgniserregend. Eine einzige klonale *E. coli* Linie, ST131-O25b:H4, repräsentiert bereits 50% aller, Breitspektrum-Beta-Laktamase (*engl.* extended-spectrum beta-lactamase (ESBL)) produzierender *E. coli* Isolate und es wird erwartet, dass diese die zukünftige Quelle pan-resistenter *E. coli* darstellt. Hier untersuchten wir mehrere Aspekte der *E. coli* ST131-O25b:H4 Linie.

Um die Pathogenität dieser Familie besser zu verstehen, haben wir zuerst das Genom unseres Modellstammes 81009, sequenziert, zusammengestellt und charakterisiert. In einem zweiten Schritt haben wir die Rolle des Komplement-Systems in Bezug auf die Protektion gegen *E. coli* ST131 Infektionen untersucht. Das Komplement-System stellt hierbei das primäre Abwehrsystem des Wirts gegen Gram-negative Bakterien dar, welche in die Blutbahn eintreten und dadurch Bakteriämie oder Sepsis auslösen. Die Untersuchungen zur Rolle des Komplement-Systems in humanem Serum ist jedoch durch eine Vielzahl anderer bakterizider Faktoren sowie der Heterogenität prä-existierender Antikörper in den Serumspendern erschwert. Um diese Probleme zu umgehen, haben wir eine neue Methode entwickelt um den alternativen und klassischen Komplementsweg mittels kommerziell erhältlicher, aufgereinigter Komplementfaktoren wiederherzustellen. Unsere Ergebnisse zeigten, dass die rekonstruierten Komplementewege aktiv waren. Ferner zeigten wir, dass die Kombination des alternativen und klassischen Komplementsweg mit einem spezifischen, monoklonalen Antikörpern (mAk) effektiv zur Elimination des 81009 Stammes führt. Die Komponenten des rekonstituierten Komplements können individuell auf das entsprechende experimentelle Modell zugeschnitten werden, wodurch wir erwarten, dass dieses auch in anderen Forschungsgebieten, abseits von Infektionskrankheiten, Anwendung finden kann. Zusätzlich haben wir eine isogene Mutante des 81009 Stammes untersucht, welche das Colistin-Resistenzgen *mcr-1* exprimiert. Während die Expression von *mcr-1* eine Resistenz gegenüber der bakteriziden als auch Lipopolysaccharid (LPS)-neutralisierenden Aktivität von Colistin induzierte, wurde die komplement-abhängige, bakterizide und anti-inflammatorische Aktivität eines LPS-bindenden mAks, nicht beeinflusst. Letztendlich haben wir die Prävalenz von ST131 Klonen unter *E. coli* Isolaten von kolonisierten, mechanisch-beatmeten Patienten einer prospektiven Beobachtungsstudie in einem einzelnen Studienzentrum evaluiert.

Zusammenfassend zeigen diese Ergebnisse, dass unabhängig von der Aufnahme neuer Resistenzgene wie *mcr-I*, der ST131 Klon effektiv durch alternative Ansätze, wie beispielsweise spezifische, bakterizide monoklonale Antikörper eliminiert werden kann.

PREAMBLE

Background

The emergence of antibiotic resistant bacteria in the last decades is a major concern, because of the limited number of novel antibiotics in development (Domalaon et al., 2018). The UK government predicts that by 2050 infectious diseases will be the main cause of death worldwide, overtaking cancer and cardiovascular problems as leading diseases. Among the bacteria, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* represent the clinically most important species exhibiting antibiotic resistance (O'Neill, 2014). Therefore, it is essential to develop new class of anti-infectives to cope with the problem of antibiotic resistance.

In this dissertation I present the results of my research activities that I carried out at Arsanis Biosciences during my Ph.D. thesis work. Arsanis Biosciences develops monoclonal antibodies (mAbs) to treat severe infectious diseases caused by antibiotic resistant bacteria. In the pipeline, the ASN200 project refers to the development of an antibody selectively targeting *Escherichia coli* sequence type (ST) 131. This clonal lineage of *E. coli* has spread all around the globe and is multidrug resistant (MDR). During my research I characterized the host-pathogen interaction in the human serum, and studies how an anti-ST131 mAb, ASN-4 could contribute to the fight against severe infections caused by *E. coli* ST131.

Innate immunity

Complement. The complement system is the first line of defense in the human blood, and it is activated by the invading pathogen as soon as it enters the blood stream. Complement activation induces inflammation and ultimately the elimination of the pathogen. The complement system is a zymogen cascade, meaning that the different factors are in the pro-zymogen form, and they must be cleaved by the upstream zymogen to get activated (**Fig. 1A**). In addition, numerous downstream molecules are cleaved by a single upstream zymogen at each step, thereby amplifying the signal. Additionally, the non-zymogen portion of the cleaved molecules may also play a role in the induction of the inflammation or on the contrary, may regulate the complement pathway itself. This result in the generation of a complex, highly regulated network in the plasma/serum.

The complement has three pathways: the alternative, the classical and the lectin pathways and all three converge to the terminal pathway (TP), leading to the assembly of the membrane attack complex (MAC) on the microorganism surface.

Alternative pathway. The alternative pathway (AP) is spontaneously activated on the pathogen surface, due to the spontaneous hydrolysis of C3 called “tick-over” yielding C3(H₂O). C3(H₂O) binds Factor B, which in turn is cleaved by Factor I in Factor Ba and Bb, forming C3(H₂O)Bb, a C3 convertase. C3(H₂O)Bb cleaves several C3 molecules in C3a and C3b, the latter binding the target surface. There, C3b is recognized by another Factor B, and again cleaved by Factor I in Bb and Ba. C3bBb is also a C3 convertase, cleaving more C3 molecules, thereby further amplifying the signal. C3bBb can be stabilized by properdin (Factor P), to increase the deposition rate of C3b and generating the C5 convertase C3b₂Bb (**Fig. 1A**) (Murphy et al., 2008).

Classical and lectin pathway. The classical (CP) and lectin pathways (LP), are induced by an initial receptor-ligand recognition on the target surface. In the case of the classical pathway, the antigen-antibody complex (or immunocomplex, IC) is bound by the C1 complex formed by C1q, C1r and C1s. The binding of C1q to IC allows the activation of C1r, which in turn activates C1s. In the lectin pathway, various lectins (including the Mannose Binding Protein, MBP) recognize specific sugar structures on the microbial surface. Analogous to C1r and C1s, upon binding to the target molecule, MBP-associated serine proteases (MASPs) get activated, inducing the lectin pathway.

Both C1s and MASPs cleave C4 in C4a and C4b, and C2 in C2a and C2b. C4b and C2b form C4b2b, another C3 convertase on the target surface. The C3 convertase cleaves C3 in C3a and C3b, C3b associates with C4b2b and forms the C5 convertase C4b2b (**Fig. 1A**) (Murphy et al., 2008).

Terminal pathway. The terminal pathway (TP) starts with the C5 convertase, which cleaves C5 in C5b and C5a. C5b attaches to the target surface through hydroxyl and amino groups, and binds C6, C7 and C8. The C5b-8 complex induces the insertion and polymerization of C9 monomers, forming the MAC. The membrane attack complex is a pore-forming complex, able to insert itself in the membrane of the pathogen, and to induce osmotic stress, lysis and death of the microorganism. Not all pathogens can be directly killed by the action of the complement; the cell wall of fungi and Gram-positive bacteria protect themselves from the membrane damage, by creating a barrier around the cell membrane therefore physically inhibiting insertion of the MAC. Nevertheless, the zymogen fragments are deposited on the

surface, and act as opsonin activating immune cells and inducing phagocytosis of these pathogens (**Fig. 1A**) (Murphy et al., 2008).

Amplification loop. The alternative pathway is also activated by the classical and lectin pathways, as C3b originated by any C3 convertases can bind Factor B and initiate the alternative pathway. Thus, the alternative pathway amplifies the signal of the other pathways increasing the number of the deposited MACs. Indeed, up to 90% of the MAC complexes induced by the classical pathway are deposited through the amplification loop provided by the alternative pathway (**Fig. 1A**) (Murphy et al., 2008).

Negative regulators of the complement system. In order to prevent destructive effects toward the host the activity of the complement system is spatially and temporally confined. It is controlled by specific activation, self-inactivation and host factors. The activation due to pathogen-associated molecular pattern (PAMP) recognition guarantees the specificity. The zymogens must bind a target nearby, otherwise they are rapidly inactivated by hydrolysis. Even so, low level of complement activation may occur in the blood targeting the host structures. Therefore, the host cells express several factors (like CR1, DAF and CD59) able to inactivate the complement on the plasma membrane. Other host factors can control the fluid phase activation of the complement. Factor H and I inhibit the alternative pathway by accelerating the decay of C3bBb and cleaving C3b (**Fig. 1A**). The classical and the lectin pathways are instead regulated by C1 inhibitor (C1-INH), as described below (Murphy et al., 2008).

Role of the C1 inhibitor (C1-INH). C1-INH is a protease inhibitor of the serpin family, a heavily glycosylated single-chain protein of 478 amino acids. C1-INH is the key inhibitor of the early components of the classical, lectin pathways, of the coagulation system, and binds certain receptors on endothelial cells as well as the lipopolysaccharide (LPS) from bacteria (Beinrohr et al., 2008).

C1-INH also has an essential role in the activation of the early components. In the blood, pro-C1r and pro-C1s form the tetramer pro-C1r₂s₂. pro-C1r₂s₂ docks itself in the conic space of the C1q and gets activated (Gaboriaud et al., 2014). However, in presence of C1-INH, C1r and C1s form the complex pro-C1rs-C1-INH₂, preventing the activation of C1 (Chesne et al., 1982). Upon binding to an immunocomplex, C1q undergoes a conformational modification, which promotes the activation of pro-C1r₂s₂ in C1r₂s₂. Afterward, C1-INH again binds and displaces C1r₂s₂ from C1, and irreversibly inhibits it. Thus, C1-INH has a dual role toward C1: it inhibits the spontaneous and non-specific activation of C1, yet allowing the activation when specific activators are present; and subsequently it “turns off” the signal (Gaboriaud et al., 2014) (**Fig. 1B**).

Loss of function or low level of C1-INH are observed in patients suffering from Hereditary Angioedema type I and II (HAE I and HAE II). These patients have an uncontrolled activation of the complement, associated with low levels of C4 and C2 (Csuka et al., 2017).

Interestingly, pathogens may use C1-INH to evade the immune system of the host. The membrane-associated virulence gene (*vg8*) of *Bordetella pertussis* binds C1-INH sequestering it from pro-C1r and pro-C1s, which leads to the activation and ultimate consumption of the complement in the fluid phase, while protecting the bacterium from its lytic action (Hovingh et al., 2017).

The interplay between the complement pathways. The three complement pathways establish a very complex network of interactions. The mannose binding protein (MBP) recognise both IgA and IgM (McMullen et al., 2006; Roos et al., 2001), and in a similar way, also the ficolin pathway can be induced by IgM (Lei et al., 2015). Also, the lectin pathway plays a significant role in the alternative pathway; Factor D is secreted in pro-form in the blood and only gets activated by MASP3 (Oroszlán et al., 2016). Recently, it has been reported that MASP1 is required for the induction of the alternative pathway induced by LPS (Paréj et al., 2018).

Moreover, not only pathogens, but also the coagulation system and apoptotic cells can induce the complement system (Merle et al., 2015a, 2015b). On the other hand, certain complement factors, like C1-INH, regulate the coagulation system (Kaplan and Joseph, 2016).

This astonishing complexity of interactions is further complicated by the presence of additional serum bactericidal factors (antimicrobial peptides, Peptidoglycan Recognition Proteins, lysozyme, galectins and others) that may act in concert with the complement or work independently against the invading pathogens (Grönroos et al., 2005; Kashyap et al., 2011; Ragland and Criss, 2017; Stowell et al., 2010; Zimmer et al., 2015). In agreement with that, the survival of a bacterium is different in serum samples from different donors, as well as different strains of a given bacterial species may survive differently in the serum of an individual (Mcintosh et al., 2015; Szijártó et al., 2017).

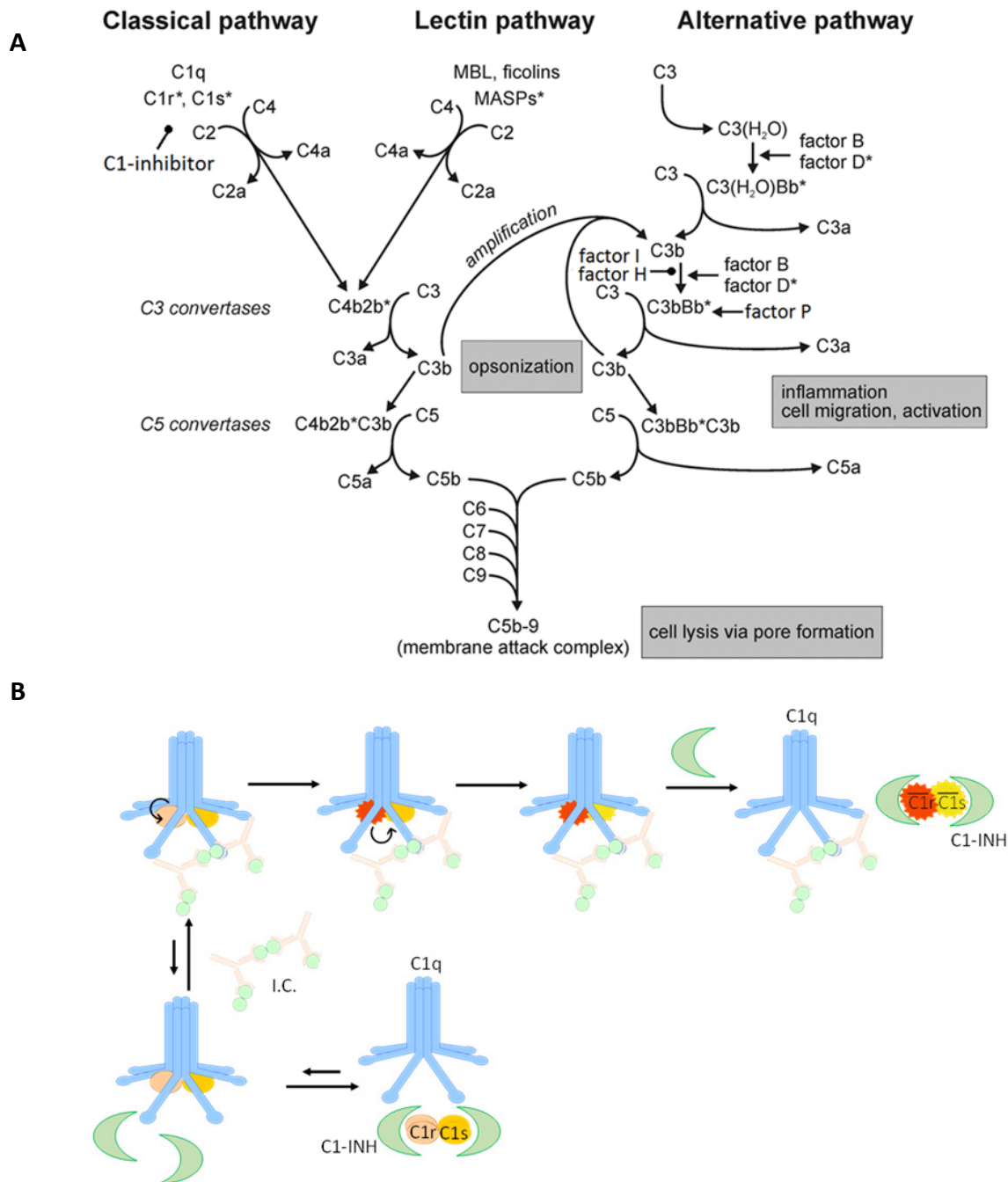


Figure 1. The complement system and the role of C1-INH. (A) Scheme of the three main complement pathways. The arrows indicate activation while the lines ending with the circle indicate inhibition. The asterisks explicit the induction of the zymogen state of the indicated protein. (B) this scheme shows the steps of activation of the C1 complex (formed by C1q in blue, pro-C1r in pale dogwood and pro-C1s in dark yellow) in presence of C1-INH (green half-moon) and an immunocomplex (I.C., formed by two antibodies in pink, bound to five antigen, in light green). The activation step is irreversible when one arrow is depicted, while double arrows indicate a reversible reaction, the favourite intermediate indicated by the longer arrow. During the process of activation pro-C1r becomes activated ($\overline{\text{C1r}}$, in red) as well as pro-C1s ($\overline{\text{C1s}}$, in yellow).

Pathogens can evade the complement. As described above, fungi and Gram-positive bacteria evade the direct killing of the complement by expressing a physical barrier to their membrane. On the other hand, Gram negative bacteria evolved many virulence factors to cope with the action of the complement. The capsule and the LPS O-antigen are expressed by almost all pathogenic strains; they act as protectins, shielding the outer membrane of the bacterium from antibodies, and complement deposition. Moreover, even in the case of recognition of these surface structures and induction of the complement, the MACs may be formed too far from the membrane to induce bacterial killing. Certain surface proteins, like OmpA, bind complement inhibitors; while factors like TraT and Iss interfere with the MAC assembly (Miajlovic and Smith, 2014).

Toll-like receptor 4 signaling. The presence of the Gram-negative bacteria in the blood is sensed also by other PAMP recognition systems beyond the complement. One of the most important is the Toll-like receptor 4 (TLR4), a transmembrane receptor recognizing the lipid A of LPS. The stimulation of TLR4 with LPS induces a strong inflammation, because of the induction of immune signalling pathways that lead to the secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF α . Similarly, to the complement system, the activation of the TLR4 does not kill the bacterium, but it is still fundamental, since it recruits immune cells and activates them. The release of LPS in the blood and recognition by TLR4 is thought to be critical in inducing sepsis. Given the essential role of the TLR4 in the immune system activation, several pathogens evolved biochemical processes to modify the LPS. Lipid A hypoacylation, or addition of molecules to the lipid A phosphates groups, are common strategies by Gram-negative bacteria to counteract the host's recognition systems (Maeshima and Fernandez, 2013; Tan and Kagan, 2014).

***E. coli* clonal lineage ST131**

Escherichia coli is a non-sporulating, Gram-negative, rod shaped facultative anaerobe bacterium. It is normally part of the microbiota in the large intestine of mammals. Unlike gut colonizing commensal strains of *E. coli*, some virulent strains are able to cause intestinal or extra-intestinal diseases. Extra-intestinal pathogenic *E. coli* (ExPEC) can cause severe diseases outside of the host's gut reservoir due to the expression of specific virulence factors (Dale and Woodford, 2015; Russo and Johnson, 2000).

E. coli isolates can be divided in four (Clermont et al., 2000; Doumith et al., 2012) to eight phylogenic groups (namely A, B1, B2, D, E, F, G and Clade 1) (Clermont et al., 2013). While group B2 and D belong to the most virulent ExPEC isolates, group A is associated with commensal strains (Clermont et al., 2000). *E. coli* strains can be further grouped into sequence types (STs) by multi-locus sequencing typing (MLST) based on the sequence polymorphism of seven housekeeping genes (Denamur et al., 2015). Compared to phylogenic grouping or capsule/LPS O-antigen serotyping, the MLST offers superior resolution and allows identification of so-called clonal lineages. Although nowadays next generation sequencing methods (NGS) offer the maximum resolution possible, MLST is still widely used and accepted, especially as MLST clustering was shown to match that of NGS (Dale and Woodford, 2015).

The ExPEC clonal lineage ST131 was first described in 2008 and has spread worldwide since then. The expansion of ST131 lineage is worrying because of its antibiotic resistance and its ability to acquire further resistance genes. This clone is generally fluoroquinolone (FQ) and trimethoprim-sulfamethoxazole resistant, and represents 40-80% of the extended spectrum beta-lactamase (ESBL) producing ExPEC isolates and a significant proportion of the carbapenem resistant *E. coli* isolates, therefore threatens with the prospect of a pan-resistant *E. coli* lineage in the future (Nicolas-Chanoine et al., 2014). Due to the slow pace of antibiotic development coupled to the fast spread of resistance, alternative, non-antibiotic based antimicrobial strategies are promising options against multi-drug resistant (MDR) bacteria.

Phylogeny and evolution of ST131. The ST131 clonal group is divided into three clades based on the *fimH* gene sequence, a type 1 pilus adhesin, required for bladder epithelial binding. (McLellan and Hunstad, 2016) . ST131 clade A is associated with *fimH*41, clade B with *fimH*22 while clade C is associated with *fimH*30 (Pitout and DeVinney, 2017). The strains in clade C are nearly exclusively FQ-resistant, and this clade branches out into H30-R and H30-Rx, the latter being associated with the presence of the extended spectrum beta-lactamase gene *bla*_{CTX-M-15} (Banerjee and Johnson, 2014). Recently, it has been reported that a new lineage of clade C1-H30R harbouring *bla*_{CTX-M-27} is arising and spreading (Matsumura et al., 2016; Merino et al., 2018), probably because of its better transmission compared to *E. coli* ST131 *bla*_{CTX-M-15} (Adler et al., 2012). However, the two lineages do not seem to be associated with specific sources of isolation, thus they do not occupy different niches (Jamborova et al., 2018). While *bla*_{CTX-M-14} and *bla*_{CTX-M-15} are found in both ST131 and non-ST131 strains, *bla*_{CTX-M-27} was so far only described in ST131 strains (Matsumura et al., 2013). Despite the isolation of ST131

strains in animals and the environment, data suggest that infections by ST131 occur exclusively in humans (Banerjee and Johnson, 2014).

ST131 strains cluster in three clades, such as clade A, B and C which is associated with *fimH41*, *fimH22* and *fimH30* respectively (Petty et al., 2014; Pitout and DeVinney, 2017). The success of *E. coli* ST131 is suspected to be caused by a combination of various events. Evidence suggests that in the 1960s and 1970s, clones of *E. coli* group B went through several insertion events of pro-phage loci (Phi 1-4, GI-Phev and Gi-LeuX) and recombination events (*parC1a* and *fimH30*), with simultaneous acquisition of an IncF2:A1:B plasmid. That background originated from the clade C0, the prototype of Clade C. The changes in the genetic landscape led to an increase in colonization capabilities, an improvement in the fitness due to the presence of the plasmid and antibiotic resistance. In particular, the presence of the *parC1a* mutation allowed the emergence of a fit FQ resistant strains in the 1980s, when the broad use of FQ began. Thus, the use of FQ induced the emergence of Clade C, which in few years (1990s) acquired several other antibiotic resistance cassettes, through transposon mobile elements inserting into the already present IncF plasmid, and accordingly separated in clades C1 and C2. IncF plasmids are prone to acquire antibiotic resistance genes and can easily propagate them among other Gram-negative bacteria. Moreover, the presence of addiction systems (*pemI/pemK* in particular) guarantees the fixation of the plasmid in the clone (**Fig. 2**) (Pitout and DeVinney, 2017).

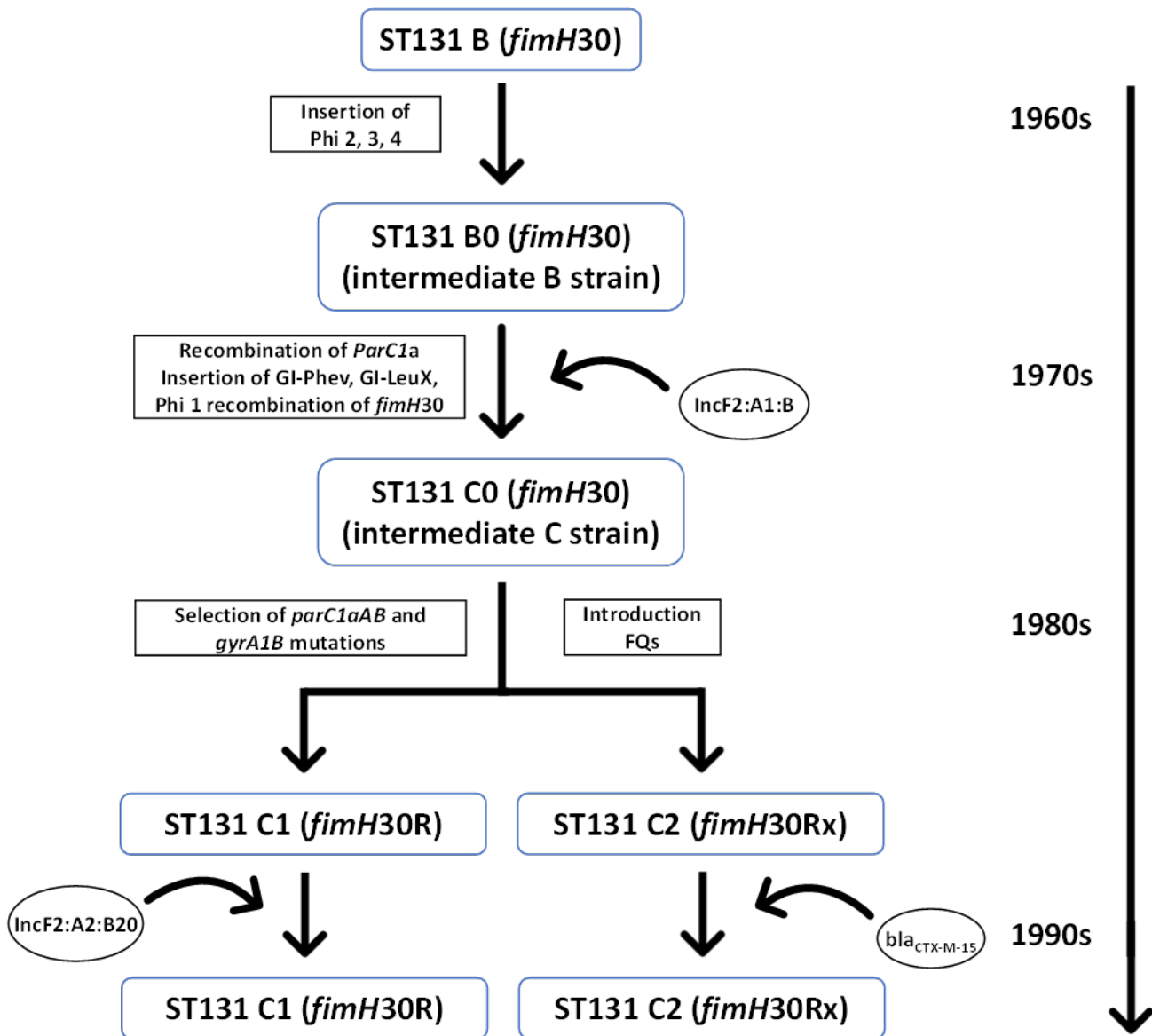


Figure 2. Step-wise evolution of *E. coli* ST131 Graphic of the proposed step-wise evolution of the *E. coli* lineage ST131 in the last decades. FQ, fluoroquinolones; GI, genomic islands; Inc, incompatibility; Phi, prophages; ST, sequence types. Figure modified from Pitout and DeVinney (2017).

Virulence of ST131 clones. ST131 strains, like most ExPEC B2 isolates have specific virulence factors (VF) compared to commensal isolates or intestinal pathogenic strains. The ST131 family can be further divided based on the set of VFs into virotype A, B, C, D and E (Nicolas-Chanoine et al., 2014). However, whether the global spread of ST131 is associated with its virulence is still under debate. Especially because epidemiological studies lack important clinical data. Moreover, several groups tested and compared the virulence of ST131 and non-ST131 strains in models difficult to translate to humans (Banerjee and Johnson, 2014). Even when the virulence of ST131 and non-ST131 or ESBL isolates is compared (Banerjee et al., 2013; Colpan et al., 2013; Johnson et al., 2010; Kudinha et al., 2013; Olesen et al., 2013), it is often performed without the consideration of the *E. coli* phylogroup; since ST131 belongs to the B2 phylogroup, it naturally carries more virulence factors than phylogroup A isolates. Indeed, in the paper of Johnson *et al.* (2010), when the virulence score of ST131 isolates was compared to that of non-ST131, mainly phylogroup B2 isolates, there was no statistically significant difference.

O-antigen O25b of ST131. The lipopolysaccharide (LPS) anchored into the outer membrane (OM) of the Gram-negative bacteria is an important virulence factor (see above). It has three main parts; the lipid A portion is embedded into the OM, the core oligosaccharide unit connects the lipid A to the outermost part, the O-antigen. The O-antigen is a polymer of oligosaccharide subunits. The number of the subunits varies between 0 and ~50 within the 1 million LPS molecules present in a single bacterial cell. The structure of the O-antigen subunit is the basis of the O-serotyping or O-type.

The O-type O25b is specifically associated with the ST131-*fimH30* clade, while O16 is expressed by clade A (ST131-*fimH41*) isolates (Banerjee and Johnson, 2014). The O25a and O25b O-antigens have been assumed to be structurally identical or related and reported as “O25” for years, since polyclonal anti-sera could not discriminate between the two (Szijártó et al., 2014). The genes encoding the O-antigen subunits are arranged into the *rfb* or *wbb* operon. The *rfb*_{O25a} and *rfb*_{O25b} are very similar, except for the genes between *wzy* and *gnd* (*rfb3* locus) (**Fig. 3A and B**). Interestingly, the *rfb3* O25a locus seems to be a derivative of the O25b due to an insertion event in the *wbbJ* gene. This modification results in the replacement of the O-acetylated rhamnopyranose (Rhap2OAc) in the O25b subunit with a N-Acetyl-Fucosamine (FucNAc) in the O25a subunit (Szijártó et al., 2014).

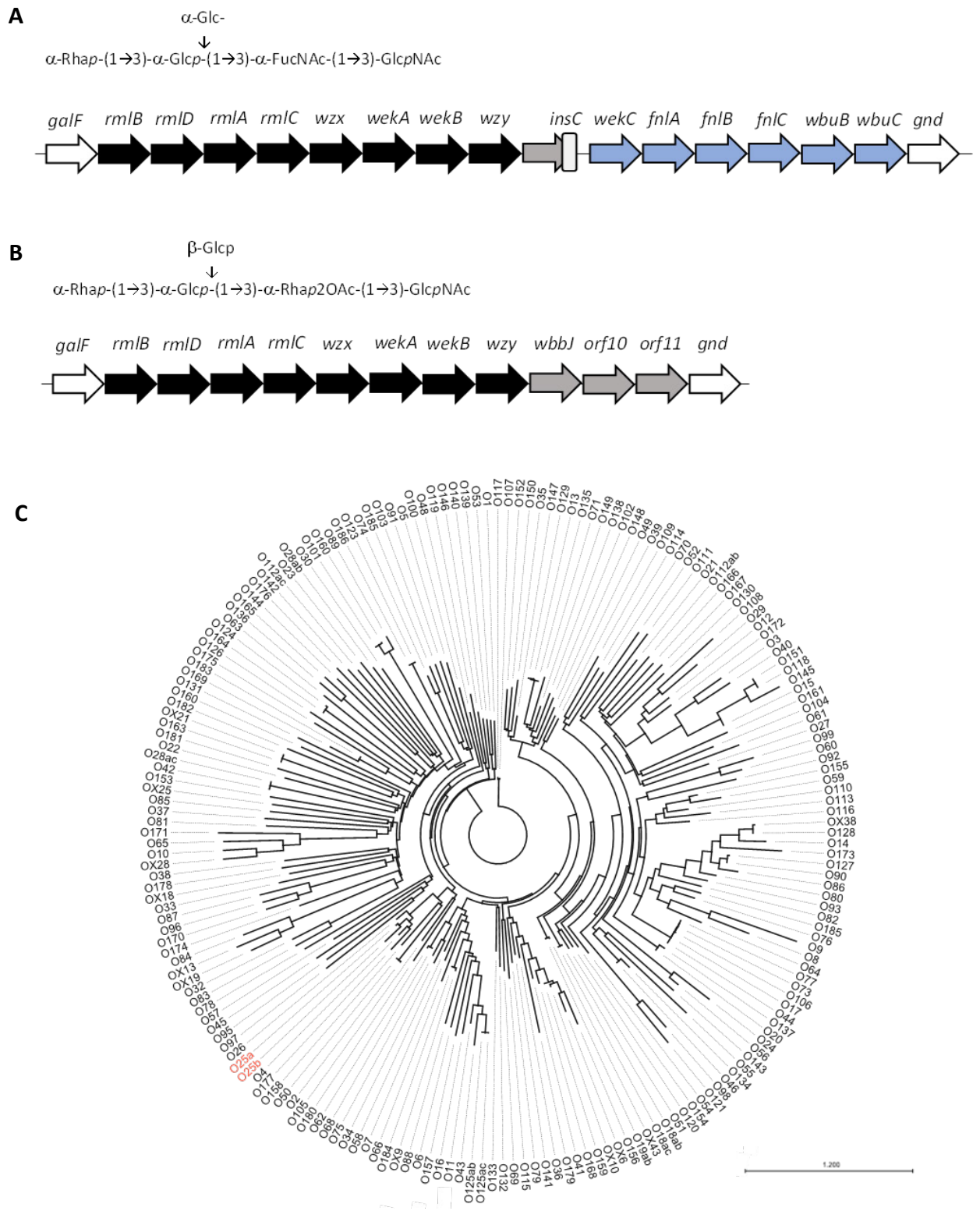


Figure 3. Structures of the O25a and O25b O-antigens. (A) Chemical structure of the O25a O-antigen (top), and genic organization of the corresponding operon. (B) Chemical structure of the O25b O-antigen (top), and genic organization of the corresponding operon. Both (A) and (B) were modified from Szijártó et al. (2014). (C) Clustering of all O-antigen operons from *E. coli*, including O25b; data elaborated from DebRoy et al. (2016).

Colistin resistance. With the recent spread of carbapenem resistance, polymyxins have been resumed as last resort antibiotics to treat infections caused by MDR Gram-negative bacteria. Polymyxins have an amphipathic cyclic heptapeptides and a lipophilic tripeptide side chain. The traditional mode of action of polymyxins is reported to be through disruption of the membrane. In particular, the cationic part of the antibiotic binds the phosphate groups on the LPS of the bacterium, displacing divalent cations. Then, the hydrophobic part insert itself in the outer and inner membrane (OM and IM), which leads to destabilization of both membranes and subsequent lysis and cell death. However, in the last years more and more evidence suggested other/additional modes of action, through vesicle-vesicle contact or hydroxyl radical production. In the first model, polymyxin acts as detergent, it induces the fusion of OM and IM, and exchange of phospholipids, which in turn impairs the lipid composition leading to osmotic imbalance and cell lysis. In the second model, polymyxin induces the production of oxygen superoxide, which is converted into H_2O_2 by the superoxide dismutase. Then H_2O_2 disrupts the Fe-S clusters, Fe^{2+} leaks from the clusters and oxides to Fe^{3+} , which through the Fenton reaction produces hydroxyl radicals, damaging DNA, proteins and lipids (Yu et al., 2015).

Several resistance mechanisms are encoded chromosomally and cannot spread to bacteria other than the daughter cells; among them, the best characterized are mutations leading to efflux pump upregulation or to a change in pathways involved in the modification of the LPS (Baron et al., 2016). However, in 2015 a group identified *E. coli* isolates from animals and meat products, expressing a colistin resistance gene, *mcr-1* from a plasmid. Mcr-1 adds a phosphoethanolamine residue to the lipid A (**Fig. 4A**), thus conferring resistance to colistin (**Fig. 4B**) (Liu et al., 2016). Very recently, it has been found that the *mcr* gene has spread globally in both animals and humans and it is diversifying (Webb et al., 2017). While *mcr-1* is not restricted to a specific clonal lineage or even bacterial species (Matamoros et al., 2017; Skov and Monnet, 2016) still the recent reports of globally disseminated high-risk clones, especially the ST131 *E. coli* clone carrying the *mcr-1* gene are of great concern.

Interestingly, *mcr-1* exhibits a certain degree of toxicity when overexpressed, increasing the fitness cost of this antibiotic resistance (Yang et al., 2017). Despite the numerous studies on *mcr-1* published up to date, there are no reports about the effect of the conferred LPS modification on TLR4 binding or on serum survival of the bacteria.

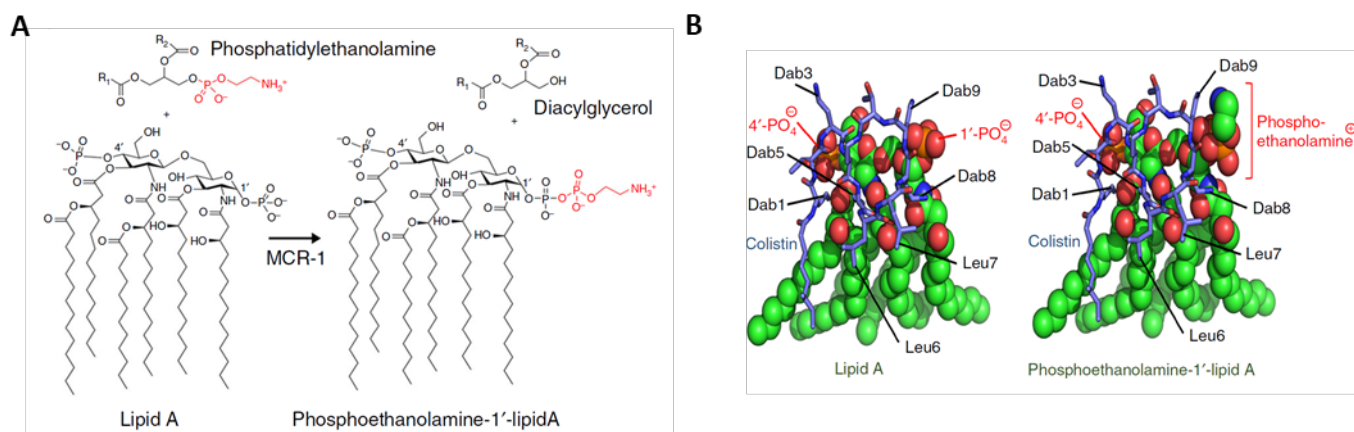


Figure 4. Chemical modification made by Mcr-1 on lipid A. (A) Chemical modification of lipid A, before and after the addition of phosphatidylethanolamine by Mcr-1, diacylglycerol as by product of the chemical reaction. (B) 3D structure of colistin bound to normal lipid A (left) or modified by Mcr-1 (right). The addition of phosphoethanolamine to lipid A lowers the 2,4-diaminobutyric acid (Dab) residues binding affinity of the polymyxins due to reduction of negative charge.

Ventilator-associated pneumonia caused by *E. coli*. The lungs are the main site of infection in patients residing in the intensive care unit (ICU). *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Acinetobacter* spp., *Klebsiella pneumoniae*, and *E. coli* are the leading pneumonia pathogens (Fihman et al., 2015; Hunter, 2012; Park, 2005). Intubation and mechanical ventilation of patients promotes lung infections, leading to ventilator-associated pneumonia (VAP). VAP is characterized by new or progressive infiltrates in the lung, purulent sputum, leucocytosis or leukopenia, and fever in patients ventilated for more than 48 hours (Johanson et al., 1972). Despite the increase of MDR *E. coli* strains in extraintestinal infections, there are only scarce studies about the prevalence of *E. coli* ST131 in patients with VAP. The papers of La Combe *et al.* (2018) and Szijártó *et al.* (2012) are the only reports describing ST131 isolates in VAP, but both without any in-depth analysis regarding patients and isolates.

***E. coli* ST131 81009 as model strain.** To study the host-pathogen interactions, we used the *E. coli* 81009 strain (PCM-2857, Mutti et al., 2018) as model organism. 81009 was isolated in the United Arab Emirates from the urine of a 76-year-old male patient (Szijártó et al., 2012). As a representative strain of the ST131 group, it expresses O25b O-antigen. 81009 carries one plasmid, pEC-81009, with three RepA proteins. Two of these proteins showed 100% homology to IncFIA and IncFII plasmid-specific replicons, while the third had 97.95% homology to IncFIB. The co-presence of different replication initiators from different incompatibility groups is possible if they belong to the same family (Villa et al., 2010). Interestingly, the search for homolog plasmids by BLAST (Altschul et al., 1990) revealed high homology (91%) to pKF3-140 from *K. pneumoniae* (FJ876827.1), although pKF3-140 does not encode for any beta-lactamase genes (Zhao et al., 2010).

E. coli 81009 is resistant to streptomycin, cephalosporins, monobactams and fluoroquinolones, but not to carbapenems, gentamycin, chloramphenicol and colistin. Additionally, the strain expresses the capsule type K5 and several virulence factors. 81009 carries virulence factors important for survival in body fluids (*issa* and *gad*), adhesion (*upaB*, *csgA*, *crl*, *fimH* and *iha*), for biofilm formation (*bscA*, *Agn43*, and F9 fimbriae), and toxins (*senB*, *sat* and *usp*). Furthermore, *E. coli* 81009 was shown to carry the *fimH30* allele, which confirms the extra intestinal pathogen status of *E. coli* 81009 (Colpan et al., 2013).

Surprisingly, we identified the genes encoding the Phd/Doc system on the plasmid of strain 81009, a well-studied toxin-antitoxin (TA) system encoded by the bacteriophage P1 plasmid. This TA system prevents the loss of the plasmid in the daughter cell (Łobocka et al., 2004), and was previously assumed not to be present in *E. coli* (Page and Peti, 2016). The

system identified in *E. coli* 81009 is located in close proximity of further phage-associated genes and transposons, indicating that the PhD/Doc system might be “imported” from the P1 phage by the means of recombination events. Beyond the aforementioned system, the plasmid also harbors the CcdBA and Kid/Kis TA systems.

As *E. coli* 81009 was used to determine the chemical structure of the unique O25b O-antigen, we analysed the *rfb/wb* operon encoding the O-antigen in more depth. Recently the phylogenic tree of 196 distinct *E. coli* O-antigen clusters was published (DebRoy et al., 2016), however the O25b O-antigen cluster was not included in this analysis. Therefore, we analysed the phylogenic relationship between the O25b O-antigen cluster of *E. coli* 81009 and all published 196 clusters (**Fig. 3C**). As expected, the O25a and O25b loci clustered together. Moreover, the O-antigen locus from O4 and O26 strains, both expressing structurally similar O-antigen subunits (Rojas-Macias et al., 2015), have in common the same parent branch (**Fig 3C**).

Passive immunization versus antibiotics to treat bacterial infections

The use of antibiotics relieved humanity from deadly infections, and allowed a significant advancement of medicine, especially in surgery. However, the often unnecessary use, and the release of antibiotics in the environment have increased the selective pressure on bacteria, leading to the selection and spread of antimicrobial resistance, and resistance genes (Hu et al., 2017).

Antibiotics are not the only weapon in our hands to fight infections. Indeed, prophylactic immunization (e.g. childhood vaccination) was the only systematic approach against infections before the introduction of antibiotics. Moreover, the use of antibiotics lead to the emergence of the resistance, while vaccination generate no or very little resistance of targeted pathogens (Kennedy and Read, 2017; Tagliabue and Rappuoli, 2018). Passive immunization with antibodies was widely used before the advent of antibiotics, however, abandoned because of the introduction of the latter (Saylor et al., 2009). The recent emergence and spreading of antibiotic resistance stimulated the renewed interest in the development of monoclonal antibodies (mAb) to treat such infections. Due to their high specificity and mode of action, they do not affect the microbiota, or bacteria outside the host. Therefore, the emergence of resistance to antibodies is less likely and slower than in case of antibiotics (Saylor et al., 2009).

While only bezlotoxumab, obiltoxaximab and raxibacumab are marketed (Calvert et al., 2018), many other mAbs against bacterial infections are being tested in Phase I or II studies

(Bassetti et al., 2017). We, at Arsanis generated a family of antibodies specific to the *E. coli* ST131 clone.

Research outline

When I joined Arsanis, a set of anti-O25b mAbs specific to *E. coli* ST131 was already generated. The anti-O25b mAbs bind with high affinity to this O-polysaccharide, and induce complement activation and phagocytosis. Due to the high affinity of the mAbs, they also stabilize the LPS micelle, thus inhibiting the release of the lipid A, thus avoiding the subsequent activation of the TLR4 pathway (Guachalla et al., 2017; Szijártó et al., 2014, 2015). However, despite thorough *in vitro* and *in vivo* characterization of the antibodies, certain aspects of the mechanism of action were not yet clarified (exact role of complement system in the bactericidal activity or the potential effect of LPS modifications on that activity). Therefore, my project was designed to investigate the host-pathogen interactions between this particular *E. coli* clone and the human serum, primarily focusing on the complement.

My first task was to determine the genomic sequences of the prototypic *E. coli* ST131 strain 81009. The sequencing and the assembly of the full genome allowed us to characterize this strain in depth, and to publish the first, fully assembled genome of an *E. coli* strain carrying the *bla_{CTX-M-27}* (**Publication I**). The whole genome sequence information also allowed the generation of several isogenic mutants of the strain 81009 and this strain became a model organism in other projects as well.

To better understand the pathogenicity of the ST131 clone and to find potential clinical indications for the use of our anti-O25b mAbs, we investigated the prevalence of this clone in patients with ventilator associated pneumonia (VAP). Therefore, we examined *E. coli* isolates from endotracheal aspirates (ETA) of ventilated patients enrolled in a prospective observational study conducted at the Lahey Clinic, in the Boston area, USA. Besides the clinical isolates, we had access to serum, ETA samples and clinical data (e.g. diagnosis and antibiotic treatments) from the patients. Thus, we could characterize the strains, the progression to VAP and the humoral immune response of the patients in depth (**Manuscript I**).

Mcr-1 modifies the lipid A of the LPS conferring resistance to colistin. Since our ST131 specific mAbs target the same molecule on the bacteria as colistin (although different part of it), we wondered whether the modification by Mcr-1 had any effect on the TLR4 response, macrophage uptake, serum survival, as well on the activity of the O25b-specific mAbs. Therefore, we addressed these questions by testing the efficacy of our mAbs against a mutant of our model strain 81009 carrying the *mcr-1* gene (**Publication II**).

As mentioned above, the anti-O25b mAbs generated by Arsanis are able to inhibit the LPS-mediated TLR4 signaling, induce phagocyte uptake and potent bactericidal activity in a complement-dependent way. However, the use of human serum as a complement source when studying the bactericidal activity of the antibody leads to biased outcome, because of the great heterogeneity among donors (mainly related to natural antibodies against *E. coli*). Thus, I generated a new tool to overcome this problem, by reconstituting the classical and alternative pathways of the complement system from purified components (**Publication III**).

PUBLICATIONS AND MANUSCRIPTS

Publication I: Mutti M, Sonnevend Á, Pál T, Junttila S, Ekker H, Galik B, Gyenesei A, Nagy G, Nagy E, Szijártó V. 2018. Complete Genome Sequence of *Escherichia coli* 81009, a Representative of the Sequence Type 131 C1-M27 Clade with a Multidrug-Resistant Phenotype. *Genome Announc* 6:e00056-18.

Publication II: Guachalla LM, Ramoni K, Varga C, Mutti M, Ghazawi A, Pál T, Nagy E, Sonnevend Á, Nagy G, Szijártó V. 2018. Retained Activity of an O25b-Specific Monoclonal Antibody against an Mcr-1-Producing *Escherichia coli* Sequence Type 131 Strain. *Antimicrob. Agents Chemother.* 62:e00046-18.

Publication III: Mutti M, Ramoni K, Nagy G, Nagy E and Szijártó V. 2018. A New Tool for Complement Research: In vitro Reconstituted Human Classical Complement Pathway. *Frontiers in Immunology*, 9: 2770.

Manuscript I: Presence of *Escherichia coli* clone ST131-O25b in patients with ventilator associated pneumonia: a prospective analysis (2018).

Submitted to *Journal of Medical Microbiology* on 12th December 2018.

Currently under revision (status Jan 27th, 2019).

Publication I

Complete Genome Sequence of *Escherichia coli* 81009, a Representative of the Sequence Type 131 C1-M27 Clade with a Multidrug-Resistant Phenotype.

Background and summary:

The clinical isolate *E. coli* 81009 is the prototypic ST131 employed by Arsanis in several experiments, and used to generate the anti-O25b mAbs. In early publications 81009 was partially characterized, but the genome sequence was not available. Thus, we sequenced the strain with two different technologies and characterised it in-depth. We found that 81009 carries several virulence factor genes and it belongs to world-wide spread bla_{CTM-X-27} group.

Personal contributions:

Processing of the genomic DNA, bioinformatic analysis, drafting of the manuscript



Complete Genome Sequence of *Escherichia coli* 81009, a Representative of the Sequence Type 131 C1-M27 Clade with a Multidrug-Resistant Phenotype

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ABSTRACT The sequence type 131 (ST131)-H30 clone is responsible for a significant proportion of multidrug-resistant extraintestinal *Escherichia coli* infections. Recently, the C1-M27 clade of ST131-H30, associated with *bla*_{CTX-M-27}, has emerged. The complete genome sequence of *E. coli* isolate 81009 belonging to this clone, previously used during the development of ST131-specific monoclonal antibodies, is reported here.

Escherichia coli strain 81009 was isolated from the urine of a 76-year-old male patient treated at Tawam Hospital, Al Ain, United Arab Emirates (1), and has recently been deposited at the Polish Collection of Microorganisms (PCM-2857). This strain belongs to sequence type 131 (ST131) and expresses the O25b O-antigen (2). *E. coli* 81009 is resistant to monobactams, cephalosporins, streptomycin, and fluoroquinolones and carries the *bla*_{CTX-M-27} allele. The recently emerging ST131-H30 isolates expressing CTX-M-27 are considered to form a new clade (C1-M27) within this clone (3, 4).

Genomic DNA of *E. coli* 81009 was extracted with the Genomic-tip 100/G kit and Genome buffer set (Qiagen) from bacteria grown in LB supplemented with 100 µg/ml ampicillin to mid-log phase (optical density at 600 nm [OD₆₀₀], ~0.5). The genomic material was sequenced on a PacBio RS II platform (Pacific Biosciences, USA) and with the Illumina HiSeq 2500 platform with a paired-end (PE) 125-bp read length (Illumina, The Netherlands).

Hierarchical Genome Assembly Process 4 (HGAP4) (5) was used for *de novo* assembly of the PacBio reads, and Illumina short reads were aligned to the *de novo* assembled genome with the Burrows-Wheeler Alignment tool (BWA) (6), followed by indel realignment and base quality recalibration with Genome Analysis Toolkit (GATK) (7). Protein-coding genes were called with Prodigal within the Prokka annotation pipeline (8). Trusted protein sequences for the annotation step were obtained from *E. coli* ST131 EC958 (9). Additional coding and noncoding RNA genes, as well as signal peptides, were predicted by RNAmmer, ARAGORN, SignalP, and Infernal within the Prokka annotation pipeline. The PlasmidFinder version 1.3 server (10) and ResFinder version 2.1 server (11) were used to identify plasmid *repA* genes and antibiotic resistance determinants.

E. coli 81009 has a circular chromosome of 5,012,084 bp and one plasmid (pEC-81009) with a length of 135,720 bp. The chromosome has a GC content of 50.8% and contains 4,987 predicted genes encoding 4,668 proteins and 319 RNAs (including 87 tRNAs, 22 rRNAs, and 1 transfer-messenger RNAs [tmRNAs]). A putative function was assigned to 91.7% of the protein-coding genes. The extraintestinal pathogen designation of *E. coli*

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81009 was supported by an array of virulence determinants identified *in silico* (12), including those involved in adhesion (*upaB*, *crl*, *csgA*, *fimH*, and *iha*), biofilm formation (*bscA*, *agn43*, and F9 fimbriae), survival in body fluids (*iss* and *gad*), and toxin production (*senB* and *sat*). *E. coli* 81009 was also shown to carry the *H30* allele of the *fimH* gene. Additionally, an 11.8-kb region (M27PP1) specific to the C1-M27 clade of the *H30* subclone (4) was identified, confirming that strain 81009 belongs to the C1-M27 clade.

Plasmid pEC-81009 was found to belong to the IncFII-FIA-FIB group by *in silico* replicon typing (10). Among the 160 predicted genes carried on this plasmid, toxin/antitoxin systems (Phd/Doc, CcdBA, and Kid/Kis) and genes encoding resistance to aminoglycosides (*aadA5*, *strA*, and *strB*), sulfonamide (*sul1* and *sul2*), tetracycline [*tet(A)*], macrolides (*mphA*), trimethoprim (*dfrA17*), and the extended-spectrum β -lactamase (ESBL) gene *bla*_{CTX-M-27} were identified.

The availability of *E. coli* 81009 in a public repository and its well-characterized phenotype and genotype make it a suitable model organism for studying the ST131 C1-M27 clade.

Accession number(s). The complete sequences of the chromosome and plasmid of *E. coli* 81009 have been deposited in GenBank under accession numbers [CP021179](#) and [CP021180](#), respectively.

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The PacBio sequencing was performed at the ETHZ/UZH Functional Genomics Center, Zurich, Switzerland.

M.M., G.N., E.N., and V.S. are employees of Arsanis Biosciences GmbH and hold shares in Arsanis, Inc., the parent company of Arsanis Biosciences GmbH. S.J., H.E., B.G., and A.G. are employees of the Vienna Biocenter Core Facilities GmbH, a member of the Vienna Biocenter, Vienna, Austria. The Illumina sequencing and the assembly and annotation of the PacBio data were done at the Next Generation Sequencing and BioComp facilities at the Vienna Biocenter Core Facilities GmbH, a member of the Vienna Biocenter, Vienna, Austria.

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Publication II

**Retained Activity of an O25b-Specific Monoclonal Antibody against an Mcr-1-
Producing *Escherichia coli* Sequence Type 131 Strain.**

Background and summary:

In the last decades, several bacterial species developed multi-drug resistance, increasing the usage of last resort antibiotics, such as colistin. However, the *mcr-1* gene, a colistin resistance gene carried by plasmids has been recently identified in *E. coli*. This colistin resistance is based on Mcr's LPS modifying activity. The LPS protects the bacterium from the action of the serum bactericidal activity and it induces inflammation via TLR4 signalling. To our knowledge, no data regarding the effects of Mcr-1 on serum survival and TLR4 induction were available.

To address this, we introduced *mcr-1* to the strain 81009 by cloning in a plasmid under the control of an inducible promoter. Although the wild-type strain carrying the resistance gene was rather serum sensitive, the expression of *mcr-1* in the 81009 background did not affect the serum sensitivity or the TLR4 activity of the colistin resistant mutant strain. Moreover, while the LPS modification affected the endotoxin neutralizing activity of colistin, the effectiveness of an O25b-antibody was not impaired, confirming that the O25b-antibody remains effective against colistin resistant ST131 isolates as well.

Personal contributions:

Plasmid preparation, generation of isogenic pairs, technical assistance for bacterial uptake and serum survival assays, performing LPS extraction, data analysis, preparation of the manuscript.



Retained Activity of an O25b-Specific Monoclonal Antibody against an Mcr-1-Producing *Escherichia coli* Sequence Type 131 Strain

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ABSTRACT Plasmid-encoded colistin resistance is emerging among extraintestinal pathogenic *Escherichia coli* strains, including those of the epidemic clone sequence type 131 (ST131)-H30. Mcr-1 transfers a phosphoethanolamine to the lipid A portion of lipopolysaccharide (LPS), conferring resistance to polymyxins. We investigated whether this modification changed the activity of the monoclonal antibody ASN-4, specific to the O25b side chain of ST131 LPS. We confirmed that, unlike colistin, ASN-4 retained its bactericidal and endotoxin-neutralizing activities and therefore offers a treatment option against extremely drug-resistant ST131 isolates.

KEYWORDS *Escherichia coli* ST131, *mcr-1*, monoclonal antibodies

Colistin (polymyxin E) and polymyxin B are considered to be last-resort antibiotics against multidrug-resistant (MDR) Gram-negative bacteria. With the spread of carbapenem-resistant pathogens, the use of colistin has increased, leading to the emergence of colistin resistance (1). Recent reports on the plasmid-mediated colistin resistance determinants, *mcr-1* and allelic variants thereof (2–4), underline the importance of transmissible resistance genes in the rapid emergence of colistin resistance (1). The acquisition of *mcr-1* by isolates belonging to globally spread MDR high-risk clonal lineages, including *Escherichia coli* clone sequence type 131 (ST131)-H30, poses a major threat to the health care system.

The gene product of the *mcr* alleles transfers a phosphoethanolamine residue to the lipid A moiety of the lipopolysaccharide (LPS) (4), i.e., the molecular target of polymyxins. The modified lipid A has much lower affinity for colistin and related polymyxins, resulting in reduced activities for these drugs. Beyond conferring resistance to polymyxins, Mcr-1 was also shown to alter the susceptibility of bacteria to antimicrobial peptides (5) and unrelated antibiotics (6). Additionally, a high level of Mcr-1 expression was reported to compromise growth rate, fitness, and outer membrane structural integrity through the integration of Mcr-1 into the membrane, as well as its enzymatic activity, i.e., the modification of the lipid A (6).

We have previously reported humanized monoclonal antibodies (7) targeting the lipopolysaccharide O25b antigen associated with the ST131-H30 clone (8), the most prevalent extraintestinal pathogenic *E. coli* (ExPEC) lineage, responsible for a wide range of infections. We demonstrated that these monoclonal antibodies (MAbs) have complement-dependent bactericidal, opsonophagocytic, and endotoxin-neutralizing activities that contribute to protection in murine models (9).

In the current study, we show that all three mechanisms of action of an O25b-specific MAb, ASN-4, were retained against *E. coli* ST131-H30 isolates producing Mcr-1. ASN-4 shares the complementarity-determining region (CDR) sequences of the previ-

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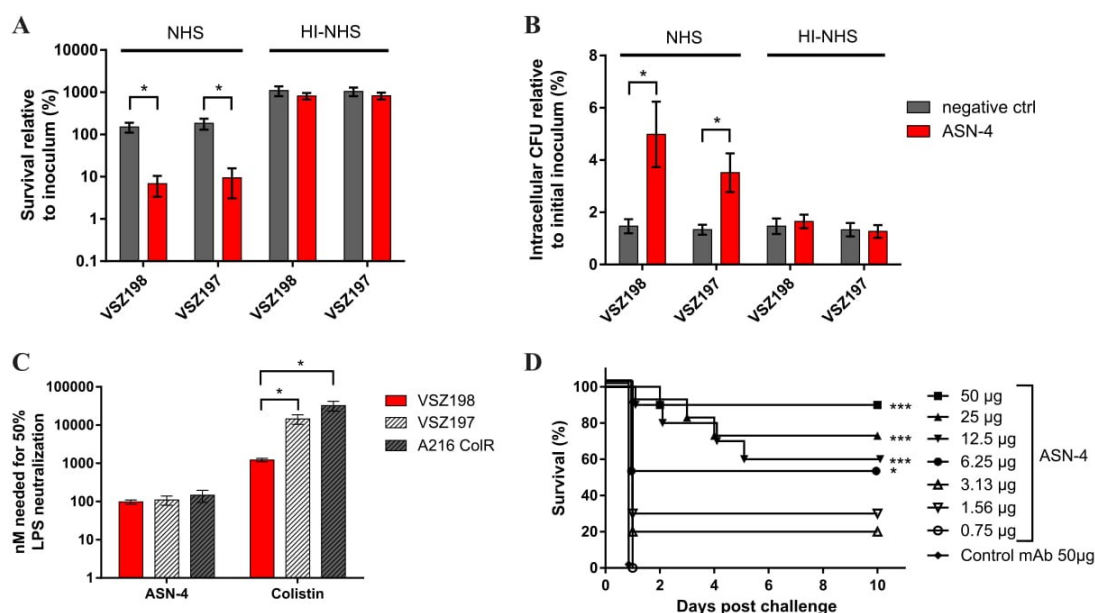


FIG 1 Activity of the ASN-4 MAb *in vitro* and *in vivo*. (A) Complement-mediated bactericidal activity of ASN-4 against an isogenic *mcr-1*-expressing (VSZ198) and -non-expressing (VSZ197) strain pair in 50% human serum after 3 h of incubation. The combined results of 3 independent experiments are shown. (B) Opsonophagocytotic uptake of the isogenic strain pair by RAW 264.7 cells mediated by ASN-4 in the presence of 5% human serum. The combined results of 6 independent experiments are shown. (C) *In vitro* neutralization of endotoxin activity of purified LPS from VSZ197 and VSZ198 or the colistin-resistant clinical isolate ABC149 as measured by a cell-based TLR4 reporter assay. The combined results of 5 independent experiments are shown. (D) Prophylactic efficacy of ASN-4 in a murine endotoxemia model. BALB/cJrj mice were immunized with the indicated doses of MAbs and subsequently challenged with 0.5 ng purified LPS extracted from strain ABC149. The results from two independent experiments with groups of 5 mice each (total of 10 mice per dose) are shown. Data shown in panels A to C are presented as the mean $\pm$ standard error of the mean (SEM), and groups were compared with Student *t* test, while on panel D, Mantel-Cox survival curves were analyzed with the log-rank test. *, *P* < 0.05; ***, *P* < 0.001. In all experiments, an isotype-matched (human IgG1) MAb with irrelevant specificity was used as a negative control. NHS, normal human serum; HI, heat inactivated.

ously published MAb A1124 (9) and therefore has identical binding specificity and affinity (with only two amino acid changes in the framework region).

To compare the potency of ASN-4 against *Mcr-1*-producing versus -nonproducing strains, an isogenic ST131-H30 pair was generated. The open reading frame of *mcr-1* was cloned from the bloodstream isolate ABC149 (10) into the *Sall* and *EcoRI* sites of plasmid pBAD322C (pBAD::*mcr-1*). Strain 81009 was transformed with pBAD::*mcr-1* (VSZ197) or with the empty vector (VSZ198). The expression of *mcr-1* was induced with 0.02% arabinose, which resulted in a 4-fold elevation in the colistin MIC in VSZ197 compared to VSZ198.

The complement-dependent bactericidal and opsonophagocytic activities of ASN-4 were measured as described previously (9). In 50% human serum as a complement source, ASN-4 mediated significant killing of both strains (Fig. 1A). Similarly, in the presence of active complement, ASN-4 significantly increased the uptake of either strain by murine macrophages (compared to a control IgG) (Fig. 1B). No significant difference was observed between the activities against *mcr-1*-expressing and -nonexpressing strains in any of these assays. These data confirm that the expression of *mcr-1* did not influence the binding of ASN-4 to the LPS O antigen or the integration of the complement membrane-attack-complex (MAC) into the structurally altered outer membrane.

The proinflammatory activity of the LPS is primarily mediated by its lipid A portion (i.e., the endotoxin). Although ASN-4 does not directly target the lipid A moiety, but rather the carbohydrate part of LPS, we were able to show that A1124, the parent MAb, neutralized the proinflammatory activity of LPS both *in vitro* and *in vivo* (9). This activity can originate from steric blocking of the lipid A binding to its receptor complex or from the stabilization of the supramolecular micelle structure of LPS that may be affected by the *mcr-1*-encoded modification. Therefore, it was of particular interest to evaluate

whether the neutralizing activity of ASN-4 against LPS altered by *Mcr-1* would be affected. The reporter cell line HEK-Blue hTLR4 (InvivoGen) was used to measure endotoxin-induced Toll-like receptor 4 (TLR-4) signaling. Cells were incubated with purified LPS extracted from VSZ197, VSZ198, or the *mcr-1*-expressing clinical ST131-H30 isolate, ABC149, in the presence of ASN-4 or the endotoxin-neutralizing antibiotic, colistin. The signal triggered by the different LPS samples alone was adjusted to be comparable. Next, we determined the MAb or colistin concentration required for 50% reduction (50% effective concentration [EC₅₀]) of the TLR-4 activation signal (Fig. 1C). While the neutralizing potencies of colistin against strains VSZ197 and ABC149 were 12- and 27-fold lower, respectively, than that against the colistin-susceptible strain VSZ198, ASN-4 had comparable LPS-neutralizing capacity against all *E. coli* ST131 strains independent of the presence of *mcr-1*.

To further corroborate this finding, we tested the efficacy of ASN-4 in a mouse endotoxemia model. Animal experiments were reviewed and approved by Arsanis' Animal Welfare and Ethics Committee and were performed according to Austrian law (BGBl. I no. 114/2012, https://www.ris.bka.gv.at/Dokumente/BgblAuth/BGBLA_2012_I_114/BGBLA_2012_I_114.html), as approved by the respective competent authority (Magistratsabteilung 58, Vienna, Austria). Six- to eight-week-old female BALB/cJ mice were sensitized to endotoxin (11), passively immunized with different doses of ASN-4 or a control MAb, and 24 h later challenged with a lethal dose of purified LPS extracted from strain ABC149. Prophylactically administered ASN-4 provided significant protection at a dose of 6.25 µg/animal (~300 µg/kg of body weight) or higher (Fig. 1D). This efficacy is comparable to that observed previously against a colistin-susceptible clinical isolate (9).

In conclusion, we demonstrate that all three mechanisms of action, i.e., complement-mediated killing, opsonophagocytosis, and endotoxin neutralization, of the O25b-specific MAb ASN-4 have been retained against an *mcr-1*-positive colistin-resistant ST131-H30 strain. Consequently, the use of such LPS O-antigen-targeting antibodies can be considered an alternative strategy to combat MDR infections, even against the emerging *mcr-1*-positive isolates.

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Publication III

**A New Tool for Complement Research: In vitro Reconstituted Human Classical Complement
Pathway**

Background and summary:

The complement system is a cascade of self-activating proteins that ultimately assemble into the so-called membrane attack complex (MAC), which lyses Gram-negative bacteria. Usually, serum is used as complement source to study the bactericidal activity of antibodies. However, due to the significant heterogeneity and presence of antibodies, assay reproducibility using human serum samples is low.

We tried to remove the pre-existing antibodies from human serum by affinity chromatography, however the method severely reduced the level of certain complement factors resulting in diminished complement activity. Therefore, we attempted to reconstitute human complement, the alternative (AP) and the classical (CP) pathways from purified complement factors. We found that the presence of C1-inhibitor in the mixture was essential to maintain a functional C1, and to generate an active CP. We further confirmed the functionality of the CP by testing the complement-dependent bactericidal activity of the anti-O25b mAb, and that of a polyclonal IVIg. Although the alternative pathway did not result in bactericidal activity, it enhanced MAC deposition induced by the reconstituted CP, and it increased the overall bactericidal activity against the ST131 *E. coli* strain. Our study represents the first report of successful *in vitro* reconstitution of the classical pathway from C1 to C9. This method offers high level of standardization and can support the study of the complement in several research fields.

Personal contributions:

Planning and performing all experiments, data analysis, drafting manuscript and preparation of the figures.



A New Tool for Complement Research: *In vitro* Reconstituted Human Classical Complement Pathway

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The complement, as part of the innate immune system, represents the first line of defense against Gram-negative bacteria invading the bloodstream. The complement system is a zymogen cascade that ultimately assemble into the so-called membrane attack complex (MAC), which lyses Gram-negative bacteria upon insertion into the outer membrane. Traditionally, serum has been used as complement source, for example to study the bactericidal activity of monoclonal antibodies or antibodies raised upon vaccination. Due to the significant donor to donor variability, as well as susceptibility of complement factors to handling and storage conditions, assay reproducibility using human serum is low. Moreover, the presence of pre-existing antibodies and antimicrobial compounds are confounding factors. To remove antibodies from human serum, we applied κ/λ -light chain specific affinity chromatography, however the method severely reduced the complement activity due to the depletion of complement components. Therefore, we attempted to reconstitute human complement—namely the alternative (rAP) and the classical (rCP) pathways—from purified complement factors. We found that adding C1-inhibitor to the mixture was essential to maintain a stable and functional C1 and thus to generate an active rCP. We further confirmed the functionality of the rCP by testing the complement-dependent bactericidal activity of a human monoclonal antibody, A1124 against an *E. coli* clinical isolate belonging to the ST131 clonal complex, and that of a polyclonal IVIg against a laboratory *E. coli* strain (MG1655) not expressing LPS O-antigen and capsule. Although the alternative pathway did not have any bactericidal activity by itself, it enhanced MAC deposition induced by rCP and increased the overall bactericidal activity against the ST131 *E. coli* strain. In conclusion, we report for the first time the successful *in vitro* reconstitution of the classical pathway of the human complement to establish a serum-free, complement dependent bactericidal assay. This system offers high level of standardization and could support the study of the complement in different research fields.

Keywords: C1-INH, bactericidal activity, monoclonal Ab, classical pathway, alternative pathway, *E. coli* ST131, MG1655, complement system

INTRODUCTION

As part of the innate immunity, the complement system is one of the first lines of defense against Gram-negative pathogens. Besides its direct bactericidal activity, the activation of the complement system stimulates phagocytosis and triggers pro-inflammatory signaling. The three different pathways—alternative, classical and lectin—converge into the terminal pathway (TP) that leads to the assembly of the C5b-9 complex, also called Membrane Attack Complex (MAC). The alternative pathway (AP) is spontaneously activated on the pathogen surface. The classical (CP) and lectin pathways (LP), are induced by an initial receptor-ligand recognition on the target surface. In case of the CP, the antigen-antibody complex (or immune complex, IC) activates the complement cascade by binding to C1, a complex formed by C1q, C1r, and C1s. In the LP, various lectins recognize specific sugar structures on the microbial surface and activate the Mannose Binding Protein-Associated Serine Proteases (MASPs) (1). Both CP and LP are negatively regulated by the C1 inhibitor (C1-INH), which sequesters and inhibits C1r, C1s, and MASPs (2). In the absence of C1-INH, C1 undergoes spontaneous activation (3, 4). Cleavage and activation of the various complement factors leads to the formation of the C5 convertase cleaving C5 to C5a and C5b, initiating the terminal pathway. C5b, attached to the target surface, associates with C6, C7, and C8 and induces the polymerization of C9 monomers, forming the MAC. MAC is a pore forming complex able to insert into lipid membranes, including the outer membrane of Gram-negative pathogens to induce membrane damage and cell lysis.

The different complement pathways interact with each other establishing a complex network in the serum (1, 5, 6). This complexity is further increased by the presence of additional serum bactericidal factors that may act in concert with the complement, or work independently against the invading pathogens (7–9). Therefore, it is difficult to dissect the contribution of the different complement pathways to the bactericidal action of the serum. It is especially relevant for studies using human serum as complement source due to the heterogeneity of the pre-existing antibody repertoires against human pathogens (10) and complement activity (11). While previous studies reported the successful reconstitution *in vitro* of the alternative pathway from individual components (12, 13), up to our knowledge, there have been no reports of a functionally active reconstituted classical pathway, particularly not to study its bactericidal activity.

We aimed to establish an *in vitro* model system with purified human complement components that allows studying complement without confounding factors.

METHODS AND MATERIALS

Bacterial Strains and Media

E. coli strains 81009 (14, 15) and MG1655 (16) were grown in LB or on Trypticase soy agar (TSA) plates (bioMérieux). To prepare overnight cultures, a single colony was inoculated in LB and

incubated at 37 °C with agitation at 200 RPM overnight. Mid-log-phase bacterial cultures were obtained by diluting the overnight cultures 1:100 in LB and incubating at 37 °C at 200 RPM until the cultures reached an OD₆₀₀ of ~0.5.

Complement Factors, Sera, and Reagents

Rabbit erythrocytes, Gelatin Veronal Buffer with or without EDTA, as well as the complement factors C3 (A113c), H (A137), I (A138), B (A135), P (A139), C5 (A120), C6 (A123), C7 (A124), C8 (A125), C9 (A126), C1 (A098), C2 (A112), C4 (A105c), and C1-INH (A140) were purchased from CompTech. Dullbecco's phosphate-buffer saline (DPBS) without (14190144) or with calcium and magnesium (14040133) was obtained from ThermoFisher Scientific. Human serum albumin (HSA) (Albion, 200 g/L) was from Biotest. The monoclonal antibody A1124, a humanized IgG1 targeting the LPS O25b-antigen of *E. coli* ST131-H30 strains, as well as an isotype-matched control mAb were expressed in CHO cells as described by Guachalla et al. (17). Blood was taken from healthy volunteers into Vacutainer® clot activator tube (367896, Becton Dickinson and Company), and the off-the-clot normal human serum (NHS) was separated by centrifugation and aliquots were stored at –80 °C until use. ClairYG® (50 mg/mL) was obtained from LFB-Biomedicaments.

Determination of Complement Activity

The complement activity was measured by commercial ELISA-based kits from Eurodiagnostica detecting C5b-9 deposition specific to classical (Wieslab COMPL CP310 RUO), alternative (Wieslab COMPL AP330 RUO) and MBP (Wieslab COMPL MP320 RUO) pathways according to manufacturer's instructions. Hemolytic activity of the complement was determined using rabbit erythrocytes according to the manufacturer's instructions using NHS as positive control.

Removal of Antibodies From Human Serum

To remove antibodies from NHS, κ/λ -light chain specific affinity chromatography was employed. The matrix was prepared by mixing three parts of κ -light chain specific beads with one part of λ -light chain specific beads (083310 and 084910, respectively, Life Technology). To avoid clogging of the resin, NHS was spin-filtered before incubating with double volume of resin at 4 °C for 10 min. The supernatant was separated from the matrix by centrifugation (Ig depleted serum, Ig-dep). As control, equivalent volume of serum was treated identically but without the chromatography matrix (mock). Both Ig-depleted and mock sera were concentrated with Vivaspın 500 (10,000 MWCO PES, Sartorius). The dilution factor was determined based on absorbance measurement at 405, 490, and 650 nm, compared to that of untreated serum. To remove beads, fibers and precipitates, the samples were spin-filtered. Bound antibodies were recovered by washing the resin with DPBS with Ca/Mg, eluting with 0.1 M glycine pH 2.0 buffer and neutralizing with 1 M Tris pH 8.0. This immunoglobulin fraction was concentrated with Vivaspın 20 (10,000 MWCO PES, Sartorius) followed by Vivaspın 500 and finally spin-filtered. In all cases Ultrafree™-MC Centrifugal Filter Devices 0.22 μ m (EMD Millipore) was employed for spin-filtration. Samples were stored at –80 °C.

Preparation of Fab Fragment of Human IVIg

Fab fragments of human IVIg (ClairYg[®]) were prepared by digestion with agarose-immobilized papain (20341, ThermoFisher) according to the manufacturer's instructions. DPBS with 10 mM EDTA pH 7.0 was used as buffer. The uncleaved Igs and Fc fragments were removed by using MabSelect Sure[®] (17543801, GE Healthcare) according to the manufacturer's instructions. The flow-through, containing the Fabs, was buffer exchanged in DPBS, concentrated (Vivaspin 500, Sartorius) and spin filtered (Ultrafree[™]-MC Centrifugal Filter Devices 0.22 μ m; EMD Millipore) to ensure sterility. An untreated aliquot of ClairYg[®] was buffer exchanged and treated the same way to be used as control. The concentration of both Fab and full IgG in DPBS was determined by bicinchoninic acid (BCA) assay (23225, ThermoFisher).

Surface Staining of *E. Coli*

To detect surface binding antibodies, 10⁸ CFU/ml bacteria were incubated with various concentration of ClairYg[®] or its Fab fragments, followed by incubation with secondary detection reagent Alexa Fluor 488 goat anti-human IgG Fc γ or Alexa Fluor 488 goat anti-human Fab₂ (109-546-170 and 109-546-097 respectively, Jackson Immuno) and stained with 5 μ M SYTO 62 nucleic acid stain (S11344, ThermoFisher). Samples were measured in a CytoFLEX flow cytometer (Beckman Coulter) and data were analyzed using the FCS Express Flow 5 (*De Novo* Software).

Immunoblotting

Samples diluted 1:400 in Laemmli buffer were separated on Mini-PROTEAN TGX 4-20% gels (4561096 or 4561094, Bio Rad) and blotted onto 0.22 μ m nitrocellulose membrane using the high MW program of the TransBlot Turbo system (Bio Rad). The membrane was blocked in 5 % skim milk in Tris Buffered Saline (TBS, Fischer scientific), incubated with 1:50,000 diluted horseradish peroxidase (HRP) conjugated goat anti-human IgG (H+L) (109-035-088, Jackson ImmunoResearch) and 1:50,000 HRP conjugated goat anti-human albumin (PA1-28334, Pierce). To detect C4 cleavage, the membrane was incubated with goat anti-human C4 serum (1:2,000, A205, CompTech) as primary antibody and with donkey IgG-HRP anti-goat-IgG (1:40,000, 6420-05, Southern Biotech) as secondary antibody. Blots were developed with Amersham ECL Prime solution reagent (GE Healthcare).

Determination of C1q and MBL2 Levels in Human Sera

Concentrations of C1q and MBL2 were determined by ELISA using a commercial kit according to the manufacturer's instructions (HK356 and HK323, respectively, HycultBiotech). The serum samples were diluted 1:200 for C1q and 1:50 for MBL2 determination. The Ig depleted sera and the eluates were diluted according to the estimated protein concentrations.

Preparation of C1/C1-INH Mixture

C1-INH was concentrated to about 2 mg/mL (Vivaspin 500, Sartorius), mixed with about 0.2–0.4 mg/mL C1 and 25 mg/mL HSA, and incubated for 10 min on ice. This mixture or C1 alone was dialyzed twice against DPBS with Ca/Mg at 4°C for 3 h followed by concentration to ~1.3 mg/mL of C1 (Vivaspin 500, Sartorius). In the C1/C1-INH mixture, the C1-INH to C1 ratio was 1.8 times the physiological ratio. The samples were spin filtered to ensure sterility (Ultrafree[™]-MC Centrifugal Filter Devices 0.22 μ m; EMD Millipore) and then aliquoted and stored at –80°C until use. At least of three different batches of C1/C1-INH were prepared and used.

Complementation Assay With C1q-Depleted Serum

C1 alone or premixed with C1-INH was incubated on ice or at 37°C for 60 min, and added to C1q-depleted serum (A300, CompTech). The activity of the classical pathway was measured with the ELISA-based kit described above.

C4 Cleavage Assay

Heat-aggregated IgG (HAGG) was generated by incubating ClairYg[®] in DPBS with Ca/Mg at 63°C for 30 min as described previously (18). C4 (200 μ g/mL), HSA (20 mg/mL) and HAGG (750 μ g/mL) were mixed on ice (final concentration indicated). C1 (67.5 μ g/mL) combined with C1-INH at various final concentrations ranging between 68.5 and 685 μ g/mL was added to the mixture. The concentration of Ca and Mg were kept constant at 0.45 mM and 0.25 mM, respectively. After 5 min on ice, the reaction was started by incubation at 37°C for 5 min. The reaction was stopped by boiling in reducing Laemmli buffer (1610737, BIORAD for 5 min. C4 cleavage was detected by immunoblotting as described above.

Reconstitution of the Human Complement

The alternative pathway (rAP) was reconstituted as reported by Schreiber et al. (12, 13). Briefly, C3, factor H and factor I were incubated for 30 min at 37°C. After cooling the mixture on ice, factor B, factor P, and the terminal pathway components C5, C6, C7, C8, and C9 were added. For the reconstitution of the rCP, C3 was incubated for 30 min at 37°C (analogous to treatment in rAP), cooled on ice, then TP components, C2, C4, and C1/C1-INH mixture were added, and then the solution was supplemented with DPBS and HSA. For the combination of the alternative and classical pathways (rAP+rCP), the alternative pathway was reconstituted as above, then TP components, C2, C4, and C1/C1-INH mixture were added, and finally the mixture was supplemented with DPBS and HSA. In all experiments the final concentration of Ca, Mg and HSA was 0.17 mM, 0.09 mM, and 25 mg/mL respectively. The proteins were added in the order described above. The final concentration of each factor matched the physiological concentration of 50% diluted human serum (3, 12): C3 at 600 μ g/mL, C5 at 36 μ g/mL, C6 at 32 μ g/mL, C7 at 27 μ g/mL, C8 at 27 μ g/mL, C9 at 29.5 μ g/mL, factor B at 100 μ g/mL, factor D at 1 μ g/mL, factor H at 235 μ g/mL, factor I at 17 μ g/mL, factor P at 10 μ g/mL, C2 at 12.5 μ g/mL, C4 at 200 μ g/mL, C1 at 67.5 μ g/mL and C1-INH at 120 μ g/mL.

Activity of rCP With or Without C1-INH

When the rCP with or without C1-INH was tested, the preparations were treated as follows. Two mixtures of rCP with or without C1-INH were prepared simultaneously as described above, on ice and aliquoted into 5 tubes. One aliquot was snap frozen in liquid nitrogen and stored at -80°C . The remaining four aliquots were incubated at 37°C or on ice for 10 min or 60 min and then snap frozen. For measuring complement activity, the aliquots were thawed on ice at once and the assay was started immediately.

Bactericidal Assay

Survival of bacteria was assayed as described previously (19). Briefly, bacteria at about $\sim 5 \times 10^4$ CFU/mL (collected from mid-log phase culture) were added to the reconstituted complement and incubated at 37°C with agitation. After 3 h, surviving bacterial count was enumerated by plating. In case of *E. coli* strain MG1655, the final concentration of each factor matched the physiological concentration in 25% diluted human serum, while in assays with the *E. coli* strain 81009, the factors were set to 50% of the physiological concentration.

Stability of rCP

rCP mixture was divided into four aliquots; one was immediately frozen at -80°C (untreated control), one underwent 3 cycles of freeze and thaw, one was stored at 4°C for 48 h, and one was incubated at 37°C for 24 h before freezing at -80°C . On the day of the testing, the samples were thawed at once and their complement activity was compared with the ELISA based kit as described above.

Statistical Analysis

Data were analyzed with paired two-tailed *t*-test using GraphPad Prism version 6. Difference was considered statistically significant if $P < 0.05$.

RESULTS

Removal of Antibodies From Human Serum Impairs the Complement

First, we tested the possibility of removing antibodies from normal human serum (NHS) to generate a complement source devoid of pre-existing antibodies for studying complement-mediated bactericidal activity. We employed affinity chromatography with Ig light chain (κ and λ) specific resin to remove all isotypes of antibodies. Based on immunoblot analysis, no IgGs could be detected in the depleted sera (Figure 1A). Next, we determined whether Ig depletion affected the complement activity of the human serum samples. The four NHS tested had comparable classical and alternative pathway activities prior to depletion, however, all had reduced or no activity after incubation with the resin (Figure 1B). We detected both MBL2 and C1q in the elution fractions together with the antibodies (Supplementary Figure 1).

Given that depletion of antibodies from NHS was not possible without compromising the complement activity, we

next attempted to reconstitute the classical and the alternative complement pathways *in vitro*.

In vitro Reconstitution of the Alternative Complement Pathway

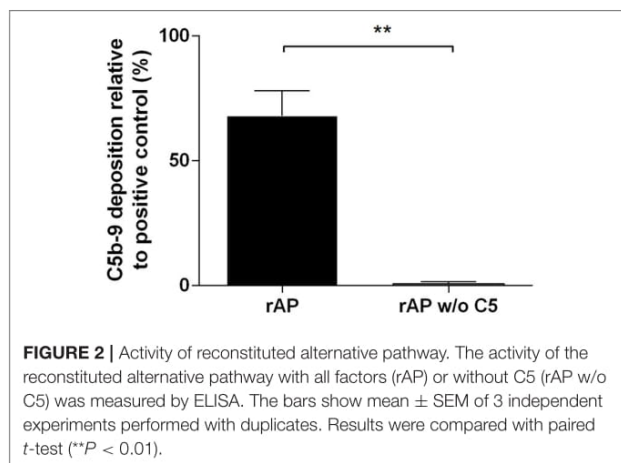
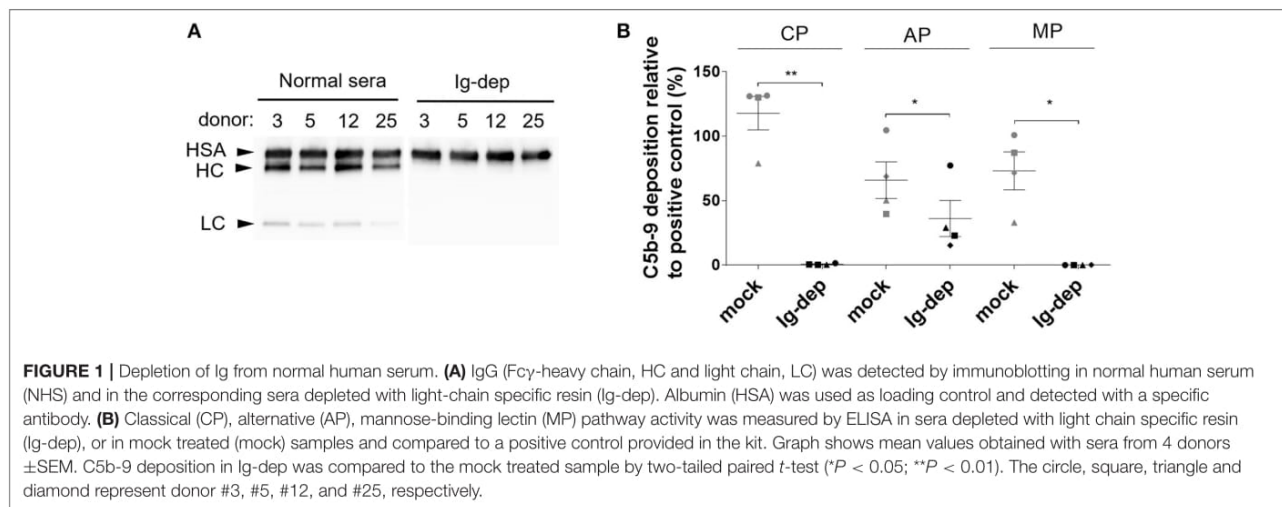
First, we wanted to recapitulate the results of Schreiber et al. (12) reported four decades ago demonstrating the functional activity of reconstituted alternative pathway (rAP). We combined purified complement components in the following order: C3, factor H and factor I incubated at 37°C , then adding factor B, factor P, and the terminal pathway components C5, C6, C7, C8, and C9. As a measure of complement activity, we detected C5b-9 assembly by ELISA (Figure 2) or by the rabbit erythrocyte lysis assay (Supplementary Figure 2). The activity of the rAP measured by ELISA was in the range of the AP activity of untreated NHS samples and the rabbit RBC lytic activity of rAP was also comparable to that of NHS (Supplementary Figure 2). The activity in both assays was specific, as it was completely lost upon the exclusion of the C5 component from the rAP mixture.

C1-INH Is Required to Preserve the Functionality of the Classical Pathway

Analogous to the generation of rAP, we attempted to reconstitute the classical pathway (rCP) by combining all factors from C1 to C9. First, C3 was incubated at 37°C , and cooled on ice before the terminal pathway components C5, 6, 7, 8, 9, then C2, C4, and finally C1 were added at concentrations equivalent to those in 50% human serum. To test the stability of the reconstituted classical pathway, the mixture was preincubated for up to 60 min at 37°C , before being assayed for the C5b-9 deposition. To resemble the physiologic activation, we measured the activity of the classical pathway with an ELISA kit, with wells coated with IgM, mimicking the immunocomplexes. Surprisingly, we detected only a very low complement activity (approximately 20% that of human serum) that was even further decreased when the mixture was incubated on ice or was completely lost after incubation at 37°C (Figure 3A).

Since the rAP, that shares many components with the CP, was functionally active and stable, we focused our investigation on the components specific for the classical pathway, particularly C1. To assess the activity of C1, we employed a C1q depleted (C1q-dep) serum preparation that lacked any CP activity and supplemented this with C1. We found that C1 was able to rescue the classical pathway activity of the C1q depleted serum, but with decreased efficiency after pre-incubation of C1 at 37°C (Figure 3B). These data suggested that the purified C1 was functional, but it lost its ability to induce the C5b-9 deposition upon incubation at 37°C .

We speculated that the loss of C1 activity was due to the spontaneous activation of C1 itself. Therefore, we assessed whether the C1 inhibitor (C1-INH), a serine protease inhibitor naturally present in human serum, would be able to stabilize C1. Indeed, we observed that the supplementation of C1q-depleted serum with C1 pre-incubated at 37°C in the presence of C1-INH, fully restored the CP activity (Figure 3B). Hence, we added C1-INH to the rCP, and the experiment was repeated as



described above. When adding C1-INH to the rCP, we observed a full classical pathway activity, in all the conditions tested (Figure 3A).

To monitor the C1 activity at the molecular level during the incubation of rCP, we detected C4 and C3 cleavage products by immunoblotting. We observed the proteolytic conversion of both C4 and C3 α chains to α' -chain in the absence, but not in the presence of C1-INH (Figure 3A). These data corroborated that spontaneous activation of C1 led to activation of the CP, which ultimately consumed the native complement factors in the mixture. On the other hand, in presence of C1-INH we did not observe any cleavage of C4 and C3, even after incubation for 1 h at 37°C (Figure 3A).

To examine whether the C1-INH concentration used in the assay still allows the specific activation of C1 initiated by immune complexes (IC), we monitored C4 cleavage triggered by heat-aggregated Ig. We found that even large excess of C1-INH (up to 10-fold the physiological C1-INH/C1 ratio) did not impair the specific activation of C1 (Supplementary Figure 3).

We also tested the stability of rCP upon freeze-thaw cycles or incubation at 37°C or at 4°C. As shown in Supplementary Figure 4, the rCP is resistant to three cycles of freeze and thaw, and stable at 4°C for 48 h, but incubation at 37°C for 24 h significantly impairs the complement activity.

rCP, Alone or in Combination With rAP, Is Functional and Induces a Strong Bactericidal Action

Next, we reconstituted the classical complement pathway in combination with rAP. Both rAP and rCP were functionally active when combined (rAP+rCP) (Figure 4A). However, when measuring the classical pathway activity in the combined system, it was lower than in the rCP alone (Figure 4A). These results suggested that components present in the rAP (partially) inhibit the classical pathway activity of rCP.

As proof of practical application, we tested the bactericidal activity of the rAP, rCP or their combination (rAP+rCP) against two different *Escherichia coli* strains in the presence of specific antibodies.

ST131 strain 81009 expressing the O25b LPS O-antigen and mAb A1124 specific for O25b (17) were co-incubated with the reconstituted complement. In presence of the rCP, we observed significant antibody-dependent bactericidal activity, while rAP alone did not support bacterial killing (Figure 4B). Interestingly, the mixture of rCP and rAP displayed enhanced bactericidal effect (Figure 4B).

Another *E. coli*, the K-12 strain MG1655, not expressing LPS O-antigen (rough), was incubated with a human IVIg preparation (ClairYg®) that was shown to contain antibodies binding to the surface of this strain (Supplementary Figure 5). In the presence of IVIg (500 μ g/ml), the MG1655 strain was completely killed in rCP or rAP+rCP, while it survived in the rAP (Figure 4C). MG1655 also survived and even grew in rCP and rAP+rCP when incubated with ClairYg®-derived Fab,

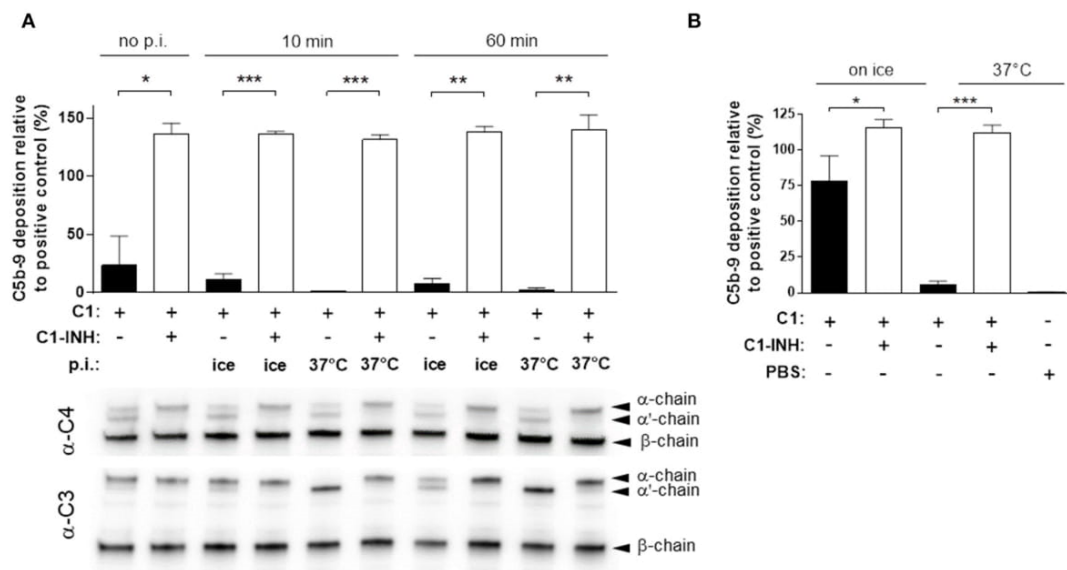


FIGURE 3 | Preserving C1 activity by C1-INH. **(A)** The activity of the reconstituted classical pathway (rCP) was measured by ELISA. rCP with or without C1-INH was pre-incubated (p.i.) for 10 min or 60 min, at 37°C or on ice, before testing for C5b-9 deposition. Graphs show mean $\pm$ SEM from 3 experiments, compared with two-tailed paired *t*-test (* P < 0.05; ** P < 0.01; *** P < 0.001). The same samples were assayed for C4 and C3 cleavage by immunoblotting that is indicated by the appearance of the α' -chain. **(B)** C1 incubated for 1 h at 37°C or on ice was added to C1q-depleted serum. The classical pathway activity was measured by ELISA detecting C5b-9 deposition. The results are expressed as % of signal compared to the positive control. Graph shows mean $\pm$ SEM from 4 independent experiments compared with two-tailed paired *t*-test (* P < 0.05; *** P < 0.001).

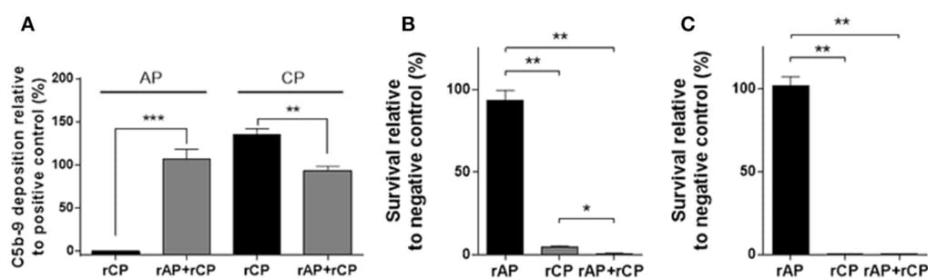


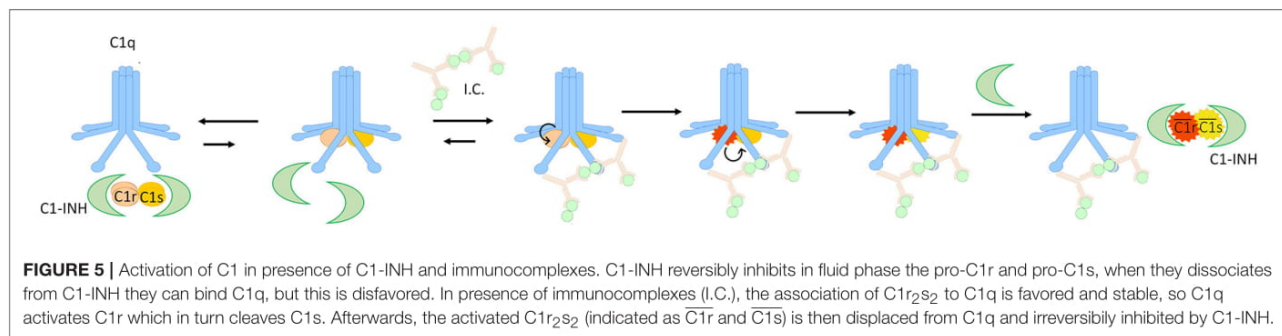
FIGURE 4 | Complement and bactericidal activity of *in vitro* reconstituted complement pathways. **(A)** ELISA detecting the alternative (AP) or classical (CP) pathway-dependent C5b-9 deposition by the *in vitro* reconstituted alternative (rAP) and classical pathway (rCP), alone or in combination (rAP+rCP). The results are expressed relative to positive control. Graphs show mean values $\pm$ SEM of 6 independent experiments analyzed with two-tailed paired *t*-test (** P < 0.01; *** P < 0.001). **(B)** Survival of the *E. coli* strain 81009 in the presence of the reconstituted complement pathways with mAb A1124 after 180 min incubation. The survival is expressed relative to the survival with an unrelated control mAb. **(C)** Survival of the *E. coli* strain MG1655 in the presence of the reconstituted complement pathways with ClairYg[®] after 180 min incubation. The survival is expressed relative to the survival in presence of ClairYg[®]-derived Fab. Graphs **(B,C)** show mean $\pm$ SEM of 3 and 2 independent experiments, respectively, analyzed with two-tailed paired *t*-test (* P < 0.05; ** P < 0.01).

corroborating that the bactericidal activity was not intrinsic to the rCP.

Low amounts of human IgG contamination was detected in all three mixtures of reconstituted complement (**Supplementary Figure 6**). Contaminants were—at least partially—introduced with the human albumin. Despite the presence of contaminating antibodies, we did not detect any specific, surface binding antibodies against the two *E. coli* strains (data not shown).

DISCUSSION

Serum bactericidal assay (SBA) is the gold standard *in vitro* assay to assess complement-dependent bactericidal activity of antibodies raised upon active immunization (10, 20) or applied as passive immunization (17). In SBA, human or rabbit serum is used as complement source and target bacteria are incubated in presence of specific antibodies or immune serum. In such assays, bacterial survival is



compared to those with a negative control antibody or non-immune serum as well as an inactivated complement source. Most often rabbit serum is preferred over human serum, because baby rabbit sera lack pre-existing antibodies (10, 20). Previous reports showed the possibility of depleting human serum from pre-existing antibodies by using Protein G affinity purification, with the drawback that C1q and C5 were significantly depleted during the process (21). Moreover, the method did not deplete IgM or IgA, two isotypes also able to activate the complement system (1, 22).

In this study, we aimed to establish a reproducible method with a complement source free of antibodies. First, we depleted human serum from all isotypes of immunoglobulins with a light chain specific resin. While we were able to remove the majority of IgGs, this treatment abolished activity of the classical and lectin pathways due to the non-specific depletion of C1q and MBL2, significantly impaired activity of the alternative pathway, and potentially depleted other complement factors by the resin.

In addition to antibodies, non-complement serum factors can also significantly contribute to the killing of bacteria in human serum (7, 8, 23). Therefore, we decided to test a bottom-up approach and reconstituted the complement system from its individual components. While reconstituted cytolytic alternative pathway was reported by two papers of Schreiber et al. ((12), (13)), up to our knowledge there are no reports of successful reconstitution of the complete classical pathway from C1 to C9, despite the significant amount of work put into reconstituting parts of it (24–31). We aimed to establish a method to reconstitute the classical pathway also in combination with the alternative pathway, to be used in studying complement mediated bactericidal activity. To make it available for the broader scientific community, we used commercially available factors purified from human serum.

First, we reproduced previous work of reconstitution of the alternative pathway (12). This was primarily done to confirm that the purified proteins were functionally active. The protocol was slightly modified with the addition of human serum albumin as carrier protein. The mixture of the AP components was active based on the detection of the C5b-9 complex measured by ELISA and the lysis of rabbit erythrocytes, and the activity was found comparable to that of human serum (32).

When we mixed the factors of the classical pathway, we detected very low CP activity. Furthermore, short incubation at 37°C (10 min) completely abolished this activity.

Apart from C1, C2 and C4, all components were already used and proved to be active in rAP, therefore we tested the activity of CP-specific factors. Importantly, the commercially available C1 is supplied with EDTA and protease inhibitors, which inhibit the activity of the complement factors, therefore, we had to remove these inhibitors by buffer exchange. This treatment could have affected activity or stability of the C1 or its components, like C1q. In line with this, C1 pre-incubated on ice was able to rescue the classical pathway in C1q-depleted serum, but not when pre-incubated at 37°C. This suggested that the buffer exchange *per se* was not responsible for the impairment of C1 activity, but rather affected its stability. Furthermore, we speculated that the lack of C5b-9 deposition in both rCP and C1q depleted serum was due to the spontaneous activation of C1r and C1s at 37°C. Indeed, in normal serum, the spontaneous activation of C1 is prevented by C1-INH that has a dual role; it inhibits irreversibly the zymogen form of C1s and C1r and in a reversible way the pro-zymogen form of C1r and C1s. In the presence of ICs, C1-INH disengages the pro-zymogens C1r and C1s, allowing their activation (4) (Figure 5). Thus, we tested whether C1-INH was able to maintain a functional C1 in an *in vitro* system as well without affecting the specific activity of C1. A previous study by Nielsen (33) showed that even large excess of C1-INH had no effect on the activation of C1 by immune complexes. However, the C1-INH preparation used in that work differed from the C1-INH available for us (34, 35). Therefore, we set up a method to detect the specific activation of C1 induced by heat-aggregated gamma globulins. We observed that even a 10-fold excess of the C1-INH did not impair the specific activation by IC.

Next, we showed that the classical pathway was fully rescued when C1q-depleted serum was supplemented with C1 co-incubated with C1-INH at 37°C. Likewise, the presence of C1-INH in rCP allowed full reconstitution of the classical pathway that was unchanged even after 1 h of incubation at 37°C. We confirmed that in the absence of C1-INH, C4 was promptly cleaved, even without specific activation. The cleavage of C4 led to the cleavage of C3 as well, but with a slower kinetics. The very low activity of the classical pathway in the rCP correlated well with the amount of native C3 in the mixture. On the other hand, when C1-INH was present, both C4 and C3 remained

uncleaved (in native state). In conclusion, the very low level of C5b-9 deposition by rCP in the absence of C1-INH is due to the consumption of the downstream factors, rather than to C1 instability. An interesting indirect proof for the requirement of C1-INH in CP is the phenotype of hereditary angioedema (HAE) type I and II. In HAE low activity of C1-INH leads to the activation of the contact system (which induces edema) and the collateral spontaneous activation of C1 and consumption of C4 and C2 (36).

While rCP appeared to remain active after three freeze-thaw cycles and at 4°C for 48 h, incubation at 37°C impaired the complement activity, in agreement with the observation by Mollnes et al. (37).

When we combined alternative and classical pathways, we found both to be active alone and in combination in the ELISA-based complement activity assay. The lower CP activity of rAP+rCP compared to rCP alone could be explained by the presence of Factor H and I. Factor H acts as cofactor for factor I to cleave C3b and C4b, and it can block the induction of the downstream cascade (5). In addition, factor H alone could also displace C1 from the ligand, as shown by Kishore and Sim (38).

After confirming the activity of the reconstituted *in vitro* complement, we proved that this experimental system is suitable for studying the complement mediated bactericidal activity of monoclonal or polyclonal antibodies. Using an extra-intestinal pathogenic *E. coli* strain 81009, we found that the rCP was able to induce a strong and specific bactericidal activity in combination with the specific mAb, A1124. The bactericidal activity was further increased when alternative and classical pathways were combined. This confirms that although the alternative pathway alone does not induce bactericidal effect, it enhances the activity of the classical pathway through the amplification loop. Of note, in the ELISA based activity assay we did not observe an increase in the classical pathway activity when rCP was combined with rAP, however this was due to the use of different complement dilutions in the two assays: in the ELISA the complement mixture was diluted 1:101 (vs. the 1:2 dilution used in the bactericidal assay) and at such a high dilution the contribution of the alternative pathway could not be detected.

We found that IVIg ClairYg® contained a significant amount of antibodies against MG1655, as also reported by others (39); therefore it was used as a source of Abs to target this strain. MG1655 is a rough and non-encapsulated K-12 archetype strain, extremely sensitive to the bactericidal activity of the serum. In

agreement with its sensitivity, the strain was completely killed in both rCP and rAP+rCP in the presence of ClairYg®. Despite of the low content of contaminating antibodies in the reconstituted complement pathways, we did not observe any killing when no ClairYg® was added to the mixture. In contrast with our data, the K-12 derivative strain, W1485, was previously shown to be killed by a reconstituted alternative pathway alone (12). However, the growth conditions used in the two studies were different, and previously also no carrier protein was added to the reconstituted mixture. Moreover, it cannot be excluded that the complement factors used previously differed in purity and potentially contained bactericidal proteins.

To our knowledge, this is the first report of the successful reconstitution of the classical pathway from individual components. In this model system, it is possible to strictly control the concentration of each component or to substitute a factor with a mutated version, as well as to supplement the assay with additional purified components, as demonstrated by adding a specific antibody. By adding lectins and MASPs to the C1/C1-INH mixture, we speculate that the system can be further complemented with the lectin pathway.

We propose that the method could have a broad applicability beyond the testing of complement mediated bactericidal activity of antibodies. It was proposed that new methods are needed to study the role of the complement in the activity of the anti-CD20 mAb, rituximab in non-Hodgkin lymphomas (40). As serum was reported to display great donor to donor heterogeneity in the killing of cancerous cells (41), the *in vitro* reconstituted complement may offer a simple method to be used in cancer studies. Similarly, this tool could be applied to study the role of the complement in viral pathogenesis (42), transplant rejection (43) and autoimmune diseases (44, 45).

AUTHOR CONTRIBUTIONS

MM performed the experiments. KR provided technical assistance. MM and VS designed the experiments. MM, VS, GN, and EN wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02770/full#supplementary-material>

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Conflict of Interest Statement: MM and KR are employees of Arsanis Biosciences. KR, GN, EN, and VS hold shares in Arsanis Inc, the parent company of Arsanis Biosciences.

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Manuscript I

Presence of *Escherichia coli* clone ST131-O25b in patients with ventilator associated pneumonia: a prospective analysis

Background and summary:

The MDR *E. coli* clonal type ST131-O25b is associated with difficult-to-treat extra-intestinal infections. However, disease progression of ST131 infections in mechanically ventilated patients has not been reported systematically.

We evaluated the presence of *E. coli* ST131 in endotracheal aspirate (ETA) samples of mechanically ventilated patients, characterised bacterial isolates and disease progression, in a prospective observational study carried out in a tertiary referral hospital in North-East USA. We found that the ST131-O25b clone was the dominant clone among *E. coli*-colonized patients. Moreover, the subjects colonized with ST131 were more likely to develop ventilator-associated pneumonia (VAP) compared to non-ST131 colonized patients. Higher levels of O25b-specific antibodies were detected in patients colonized by O25b *E. coli*, already early during ventilation, suggesting that these patients had a prior exposure to this clone through colonization/infection. Importantly, these antibodies mainly belonged to the IgG₂ sub-class that has the lowest complement activating capacity among the human IgG sub-class, and may explain why most colonized patients developed infection despite having high anti-O25b titers. Our results suggest that monitoring high risk-patients for colonization with multi-drug resistant bacteria like *E. coli* ST131 O25b could be beneficial, to achieve higher success rates of antibiotic therapy and detect patients at high risk for VAP.

Personal contributions:

Performing genetic characterization, data analysis.

Confirmation of manuscript submission:

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Presence of Escherichia coli clone ST131-O25b in patients with ventilator-associated pneumonia: a prospective analysis --Manuscript Draft--

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Abstract:	Purpose: To evaluate the presence of E. coli ST131-O25b in endotracheal aspirate (ETA) of mechanically ventilated patients and assess patient and pathogen characteristics. Methodology: We studied mechanically ventilated patients colonized by E. coli from a prospective observational study in a tertiary medical center in the USA. Specific-antibody titers and clinical characteristics of patients were evaluated. Results: E. coli was isolated in 13 of 125 patients (10.4%) colonized by potentially pathogenic bacteria in the trachea. In 7 of these cases, the ST131-O25b clone was identified. While 5 of 7 ST131-O25b colonized patients developed VAP, only one of 6 non-ST131 E. coli colonized patient presented pneumonia. All but one ST131-O25b colonized patients were recruited from the medical ICU, while 5 of 6 non-ST131 colonized patient were admitted to the surgical ICU ($p = 0.029$, $OR=30$). Already early during ventilation, higher O25b specific IgG and IgA titers in ETA ($p = 0.01$) and an increase in anti-O25b plasma IgG2 titer was measured in patients with O25b E. coli compared to non-O25b E. coli colonization Conclusion: We detected a significant proportion of ST131-O25b among E. coli isolates from tracheal-colonized patients particularly those progressing to VAP. The specific systemic and mucosal titer even prior first detection of the pathogen in the ETA suggests preceding exposure of the patients to this clone.
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Presence of *Escherichia coli* clone ST131-O25b in patients with ventilator-associated pneumonia: a prospective analysis

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Keywords:

Ventilator-associated pneumonia, *E. coli*, antibiotic resistance, nosocomial infection, ST131-O25b

Abstract

Purposes: To evaluate the presence of *E. coli* ST131-O25b in endotracheal aspirate (ETA) of mechanically ventilated patients and assess patient and pathogen characteristics.

Methodology: We studied mechanically ventilated patients colonized by *E. coli* from a prospective observational study in a tertiary medical center in the USA. Specific-antibody titers and clinical characteristics of patients were evaluated.

Results: *E. coli* was isolated in 13 of 125 patients (10.4%) colonized by potentially pathogenic bacteria in the trachea. In 7 of these cases, the ST131-O25b clone was identified. While 5 of 7 ST131-O25b colonized patients developed VAP, only one of 6 non-ST131 *E. coli* colonized patient presented pneumonia. All but one ST131-O25b colonized patients were recruited from the medical ICU, while 5 of 6 non-ST131 colonized patient were admitted to the surgical ICU ($p = 0.029$, $OR=30$). Already early during ventilation, higher O25b specific IgG and IgA titers in ETA ($p = 0.01$) and an increase in anti-O25b plasma IgG2 titer was measured in patients with O25b *E. coli* compared to non-O25b *E. coli* colonization

Conclusion: We detected a significant proportion of ST131-O25b among *E. coli* isolates from tracheal-colonized patients particularly those progressing to VAP. The specific systemic and mucosal titer even prior first detection of the pathogen in the ETA suggests preceding exposure of the patients to this clone.

Introduction

The worldwide spread of multi-drug resistant (MDR) Gram-negative pathogens poses a significant threat to the health-care system [1, 2]. In patients treated in intensive care units (ICUs), the respiratory tract is a common site of infection [3, 4]. An increase in Gram-negative bacterial infections was observed among ICU patients, including those developing ventilator-associated pneumonia (VAP) [4–6]. An estimated 8 to 28% of intubated patients develop VAP [7], and this is associated with longer intubation time and an attributable mortality rate close to 13% [8, 9].

Since its first description in 2008, the *E. coli* sequence type (ST) 131 has become globally distributed and emerged as one of the most prevalent multidrug resistant *E. coli* lineages associated with extra-intestinal infections [10]. Although recovery of *E. coli* ST131 from the respiratory tract has been investigated in hospitalized patients in several states of the Asian continent [11], to the best of our knowledge, no such study has been systematically pursued in Europe or in the USA. To fill this gap, we examined *E. coli* isolates recovered from the endotracheal aspirate (ETA) samples of mechanically ventilated patients. The *E. coli* ST131 clone was detected in several subjects and is being described in our study below. Here we describe for the first time the systematic detection of the *E. coli* ST131 clone in tracheal aspirates of patients undergoing mechanical ventilation and its association with VAP in a single-center, prospective observational study.

Patients and methods

Patient population and sample collection

Patients admitted between June 2014 and June 2015 to three ICUs at the Lahey Clinic/Tufts University (Burlington, MA, USA) were included in the analysis. Clinical and demographic data were collected in de-identified form. VAP was retrospectively assigned based on clinical criteria defined by Johanson *et al* [12].

Endotracheal aspirate (ETA) samples were obtained from patients by suctioning through the endotracheal tube as part of the standard care. Specimens were analyzed using standard microbiology techniques, including semi-quantitative plating on non-selective media. When *E. coli* was recovered from the ETA, single colonies were preserved.

Plasma samples were collected using plasma separator tubes (PST). All plasma and ETA samples were stored at -80°C or on dry-ice. The study protocol was approved by the Institutional Review Board of Lahey Clinic. Informed

consent was waived, since only leftover materials were used, and no additional intervention or change in treatment plan was implemented.

Antimicrobial susceptibility

The first *E. coli* isolate of tracheal-colonized patients was subjected to antibiotic susceptibility testing using E-Test® strips (bioMérieux, Vienna, Austria) according to the manufacturer's instructions. The results were interpreted according to published EUCAST breakpoints tables (version 8.0). MDR was defined as resistance to at least three antibiotic classes [13].

MLST and phylogenetic typing of *E. coli* isolates

The multilocus sequence type (MLST) of the first and last *E. coli* isolates of each patient was determined as previously described [14] and the alleles were assigned according to the Achtman's scheme (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). For the amplification of the MLST alleles, genomic DNA was isolated using the DNA extraction kit (Qiagen) and PCR was performed using the Phusion (Thermo) and GreenTaq polymerase (Promega). The phylogenetic groups were determined by PCR [15]. O25b typing was performed according to Blanco et al [16].

Virulence factor determination

Selected virulence factors were detected by PCR using specific primers listed in the Suppl. Table 1.

Determination of O25b LPS specific antibody levels in ETA and plasma samples

Details for the antibody determination in ETA and plasma samples are provided in the online data supplement.

Statistical analysis

Statistical analyses were performed using Graphpad Prism software version 6.07. Quantitative variables were analyzed using the Mann-Whitney *U* and unpaired t-tests. Qualitative variables were analyzed using the Fishers's Exact test. Where required, Odds Ratios were calculated by adding 0.5 to each value.

Results

Presence of *E. coli* ST131 isolates in ETA samples of ventilated patients

250 patients admitted to the medical or surgical ICUs between June 2014 and June 2015 that were mechanically ventilated for at least two days were enrolled in the study. After exclusion of patients with incomplete clinical information, data of 240 patients were analyzed in this study (Fig. 1). 125 of 240 patients exhibited bacterial colonization of the trachea (52.1%).

E. coli was isolated from ETA samples of 13 patients (Fig. 1 and Suppl. Fig 1). MLST typing revealed that the ST131 clonal lineage was the single most prevalent sequence type present in 7 patients (Table 1). Among the non-ST131 strains (n=6), three belonged to ST73, one to ST69 and one to ST1193. In all *E. coli*-colonized patients the first and last isolates belonged to the same sequence type, therefore only results with the first isolate are shown in Table 1.

All *E. coli* ST131 strains were positive with the *rfb*_{O25} PCR and all but one (LEC_004) expressed the O25b O-antigen as verified by ELISA (data not shown) using a specific O25b antibody [17]. Strain LEC_004 was shown to express rough LPS by immunoblot, i.e. did not express the O-antigen (data not shown).

Interestingly, the only ST69 strain, LEC_003 carried the *rfb*_{O25b} locus and expressed the O25b antigen. The phylogenetic group B2 was dominant among non-ST131 isolates, where groups A and D were represented by one isolate each.

Clinical characteristics of patients colonized with *E. coli*

While 6 of 7 ST131 colonized patients were admitted to one of the two medical ICUs of the hospital, 5 of 6 patients carrying non-ST131 *E. coli* strains were from the surgical ICU (Table 2; $p = 0.029$, Odds ratio [OR]=30). Respiratory failure or pneumonia was reported as admission diagnosis in 5 patients with ST131 tracheal colonization (Table 3). On the other hand, none of the non-ST131 colonized patients were admitted with respiratory conditions. When VAP was retrospectively assigned, only one of the 6 non-ST131 *E. coli* carriers had VAP (17%), whereas five patients carrying ST131-O25b developed VAP (Table 2; $p = 0.08$, OR = 25). *E. coli* colonization preceded VAP by 1-3 days in all but one patient (Suppl. Fig. 1). Tracheal colonization by other bacterial pathogens (co-colonization) was observed in 10 of the 13 *E. coli* colonized patients. The most frequent pathogens were *S. aureus* (5 patients) followed by *K. pneumoniae* (2 patients), *P. aeruginosa*, *P. mirabilis*, *H. influenza*, and *K. oxytoca* (1-1 case, Suppl.

Fig 1). The presence of two pathogens in the same ETA sample collected during the time when VAP diagnosis was made, only occurred in a non-ST131 colonized patient (Subject ID-210, Suppl. Fig. 1).

The duration of hospitalization was significantly longer for the non-ST131 compared to the ST131 colonized patients (26 days vs 15 days, $p=0.01$; Table 2) irrespective of VAP. No differences were detected in the clinical outcome of patients colonized with ST131 compared to those with non-ST131 strains.

All *E. coli* colonized patients received antibiotics while ventilated; vancomycin was the most often used (12 patients) followed by cephalosporins (11 patients) and fluoroquinolones (7 patients).

Antimicrobial susceptibility and virulence factor profile of colonizing *E. coli* strains

To evaluate the potential role of antibiotic resistance and selected virulence factors in the success of the ST131 clone colonizing the respiratory tract of mechanically ventilated patients, the antibiotic susceptibility and virulence profile of the *E. coli* isolates was tested (Table 4, 5, Suppl. Tables 2, 3). Among the non-ST131 strains, the ST1193 strain was the only MDR isolate and 3 strains were susceptible to all antibiotics tested (Suppl. Table 3). Conversely, all ST131 isolates were resistant to fluoroquinolones and 4 out of 7 ST131 isolates were MDR (Table 5, Suppl. Table 3). It is important to note that the resistance profile of *E. coli* isolates from VAP patients varied greatly and that almost half of the patients not progressing to VAP were colonized with MDR *E. coli* strains. No specific virulence factors were identified in association with the ST131 strains or with strains from VAP patients (Table 4). Interestingly, isolates from patients developing VAP showed a virulence score of 3.5 on average, which was slightly lower (although not statistically different) than the average virulence score in isolates from non-VAP patients (4.3, Suppl. Table 2).

Anti-O25b antibody profile of *E. coli* colonized patients

The ST131 *E. coli* clonal lineage express the characteristic O25b LPS antigen on their surface [18]. We determined specific antibody titers against this antigen in plasma and ETA samples of patients colonized with *E. coli*. The plasma samples were taken from the patients at the beginning of mechanical ventilation (median = 1 day after intubation, range 0-9 days; Suppl. Fig. 1), in 8 patients prior to the detection of *E. coli* in the ETA. In patients colonized with O25b-expressing *E. coli* (all but one ST131 isolate and the ST69 strain), we observed a trend for

higher O25b-specific IgG2 and IgM titers, however the differences were not statistically significant (median titers for IgG2 44.4 vs 1.9 and for IgM 11.2 vs 3.5 $\mu\text{g/mL}$ respectively; Fig. 2).

We also determined O25b LPS-specific IgA and IgG antibodies in the first ETA sample available from each patient (median 1 day after intubation, range 0 to 14 days). O25b specific antibody titers of both classes were significantly higher in the ETA samples of patients colonized with O25b-expressing *E. coli* compared to non-O25b colonized patients ($p = 0.01$, Fig. 3).

The presence or absence of IgG in ETA samples might suggest respectively either the leakage of systemic antibodies into the epithelial lining fluid due to tissue disruption or dilution of the epithelial lining fluid (ELF) beyond detection limit during sample preparation. Importantly, dilution of the ETA samples (relative to ELF) is individual and variable during the ETA sampling procedure, making the results potentially incomparable among patients. To correct for the potential dilution effect, we measured the urea concentration in the plasma and ETA samples to normalize to ELF volume [19]. Even upon normalization of antibody titers to ELF volume, we observed significantly higher specific IgG titers in the ETA of patients with O25b *E. coli* (Suppl. Fig 2).

Discussion

The increased prevalence of MDR Gram-negative infections in the ICU and in particular among VAP patients justifies specific interventions in predicting, detecting, and treating patients at risk. Systematic surveillance of colonization in mechanically ventilated patients might not only predict MDR associated VAP, but also support antibiotic selection in associated infections [20, 21].

We studied *E. coli* isolates colonizing the trachea of mechanically ventilated patients in a prospective, single-center observational study. We found that the ST131 clone was the single dominant sequence type representing more than half of all colonizing *E. coli*. Notably, among *E. coli* isolates from patients developing VAP, this high-risk clone was nearly exclusive.

Although previous studies have shown the association between certain virulence factors and VAP [22], we could not find any factors specific to the ST131 group or to isolates from VAP patients. However, we observed a clear difference in the antibiotic resistance profile of the ST131 vs non-ST131 isolates. Importantly, all ST131 isolates were resistant to fluoroquinolones and 4 out of 7 were non-susceptible to 3rd-4th generation cephalosporins, both antibiotic classes often used in the ICU patients (Suppl. Fig. 1).

The antibiotic susceptibility profiles of the colonizing strains also reveal that some antibiotics that patients receive as standard of care (e.g. levofloxacin) are inappropriate/ineffective against circulating high-risk clones. Five of 7 patients with ST131 colonization received fluoroquinolones that otherwise are ineffective against the corresponding strain. However, it has to be noted that the patients received antibiotics for other underlying reasons than MDR colonization. In contrast, no such difference in resistance pattern could be observed between *E. coli* isolates from patients that did develop VAP versus those that did not. This could indicate that, while resistance of *E. coli* ST131 to antibiotics might contribute to its dominance among colonizing strains, antibiotic resistance is likely not the sole factor driving progression to VAP.

We found that despite the higher progression rate to VAP, ST131 colonized patients were hospitalized for a significantly shorter period than those colonized with non-ST131. This result is in contrast with previous reports on VAP increasing the length of hospitalization [23]. However, it is important to note, that in this study there was a significantly longer hospitalization observed in patients recruited from the surgical ICU, where all but one non-ST131 patients were admitted (data not shown). Therefore, the observed difference in duration of hospitalization between ST131 and non-ST131 colonized patients might be due to other underlying conditions.

When compared with non-O25b *E. coli* colonized patients, the plasma and ETA of O25b colonized patients displayed increased specific antibody levels on the day or even before detecting tracheal colonization with *E. coli* ST131. Previous exposure of the O25b-colonized patients to this specific antigen might be a possible explanation. The ST131 clonal group was reported to be an efficient colonizer of the mammalian gut [24]. To our knowledge, there are no reports on the antibody response elicited by intestinal colonization. Our study was not designed to monitor potential fecal carriage of this clone, but it is tempting to speculate that intestinal carriage of this clone was responsible for the higher pre-existing O25b titers.

In agreement with previous findings on carbohydrate antigens [25], we observed that the O25b-specific antibodies are restricted to the IgG2 isotype. Similar anti-LPS IgG2 antibodies were recently reported in patients developing *E. coli* urosepsis [26]. While antibodies usually protect hosts from infection, these were shown to have the opposite effect by inhibiting serum mediated bacterial killing. Analogously, high IgG2 titers against *Pseudomonas aeruginosa* LPS prevented clearance of infection in patients with bronchiectasis [27]. These results imply that a biased antibody response generating high titer of IgG2 antibodies against LPS from Gram-negative bacteria could be detrimental instead of beneficial. Whether high O25b-specific IgG2 titers may predispose to extra-

intestinal colonization and/or progression to invasive infection by O25b-expressing *E. coli* strains warrants future investigation. Notwithstanding, an LPS-specific IgG1 antibody against O25b-ST131 bacteria was observed to be protective in *in vitro* and *in vivo* animal models [28]. However, a potential application of such antibodies against pneumonia remains to be investigated.

The major limitation of our study is that it was performed in a single center and included a small number of *E. coli*-colonized patients. These patients were often co-colonized with other pathogens and were heterogeneous at their admission, treatment and clinical outcome; making it difficult to analyze the contribution of *E. coli* ST131 in their clinical evolution. Moreover, no fecal samples were available for carriage analysis.

In conclusion, analysis of prospectively collected ETA samples of mechanically ventilated patients revealed high percentage of *E. coli* ST131 isolates that were characterized by the resistance to multiple classes of antibiotics. Patients colonized with this strain were more likely to develop VAP when compared to patients colonized with non-ST131 clones. Colonization and/or infection with this clone was associated with the presence of antibodies of IgG2 class. These results further highlight the success of this clone in colonizing and infecting humans and emphasize the importance of its identification and monitoring to achieve higher success rates of antibiotic therapy.

Figures

Figure 1 Flowchart of the study enrollment and analysis

Figure 2 O25b specific antibody concentration in plasma of patients colonized with O25b expressing (+) and non-expressing (-) *E. coli* strains. The concentration was calculated based on a standard curve determined with an O25b specific mAb in the corresponding isotype format. Middle values represent median. Titers of patients with O25b expressing and non-expressing strains were compared with the Mann-Whitney *U* test. # patient carrying the O25b expressing ST69 strain, § patient carrying the ST131 strain with rough LPS phenotype (no O-antigen).

Figure 3 O25b specific antibody concentration in ETA of patients colonized with *E. coli*. Plots show median and individual antibody titers of A) IgG (µg/mL, upper panel) and B) IgA (arbitrary units). Graphs show results of one experiment run in duplicates. Subject 211 was excluded from the analysis on panel B, as no titration was observed in the ELISA due to very high IgA titers. The Mann-Whitney *U* test was used for statistical analysis. # Patient carrying the ST69 strain expressing the O25b antigen. § Patient carrying the ST131 strain with a rough O25b phenotype.

Conflict of interest

LG, GN, EN and VS are inventors on patents covering monoclonal antibodies against *E. coli* ST131 and may hold shares in Arsanis, Inc., the parent company of Arsanis Biosciences GmbH. The authors declare that they have no other conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Contributions

LG planned, performed and analyzed experiments; MM and KR genotyped virulence factors and sequence types; GN analyzed hemolytic activity profiles; EK, VS and LG analyzed and interpreted clinical data; EN, ZM, contributed to the hypothesis and design of the study, JH identified patients and provided samples, BW analyzed antibiotic susceptibility profiles, LG, VS, GN, ZM, EN wrote the manuscript; VS conceived the study. All authors reviewed the manuscript.

Appendix A. Supplementary tables, materials and figures can be found in appendix A (online version)

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341

Figure 1

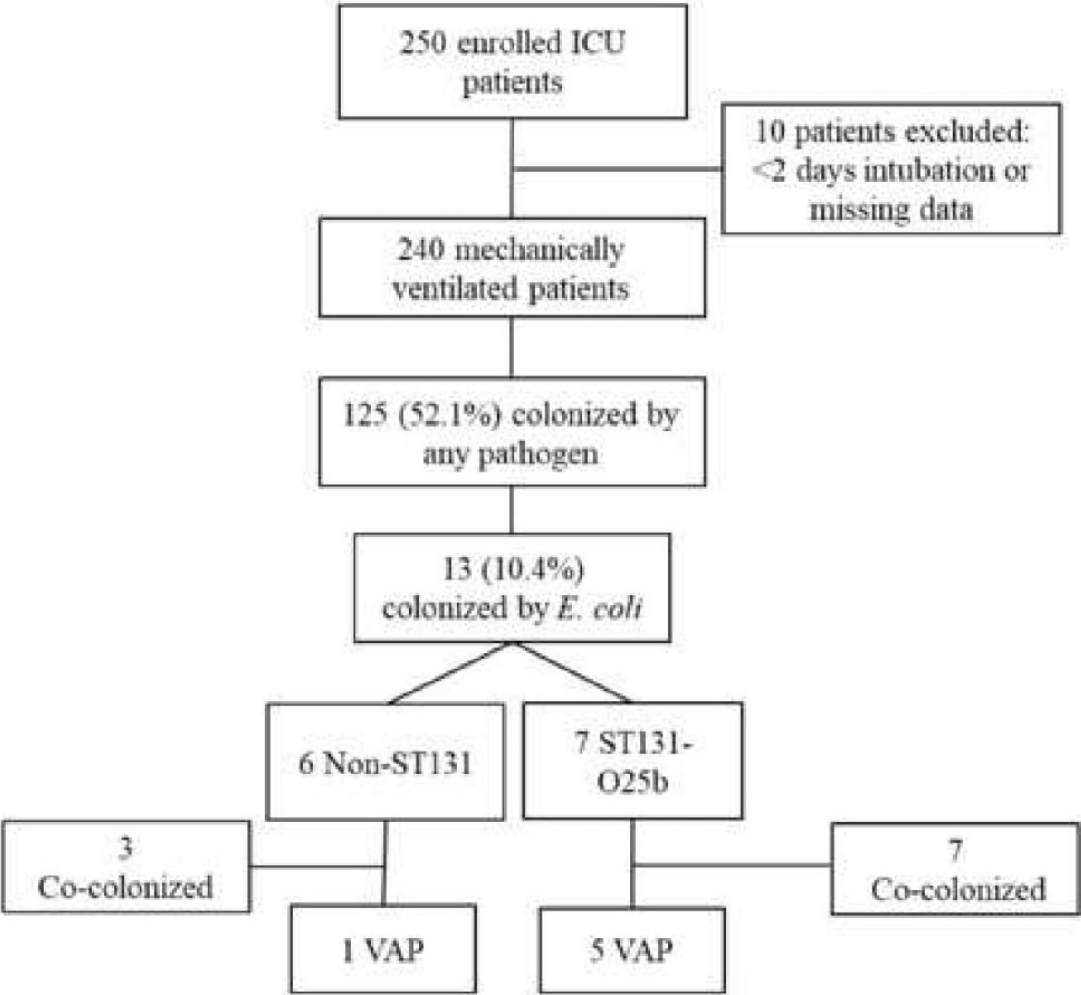


Figure 2

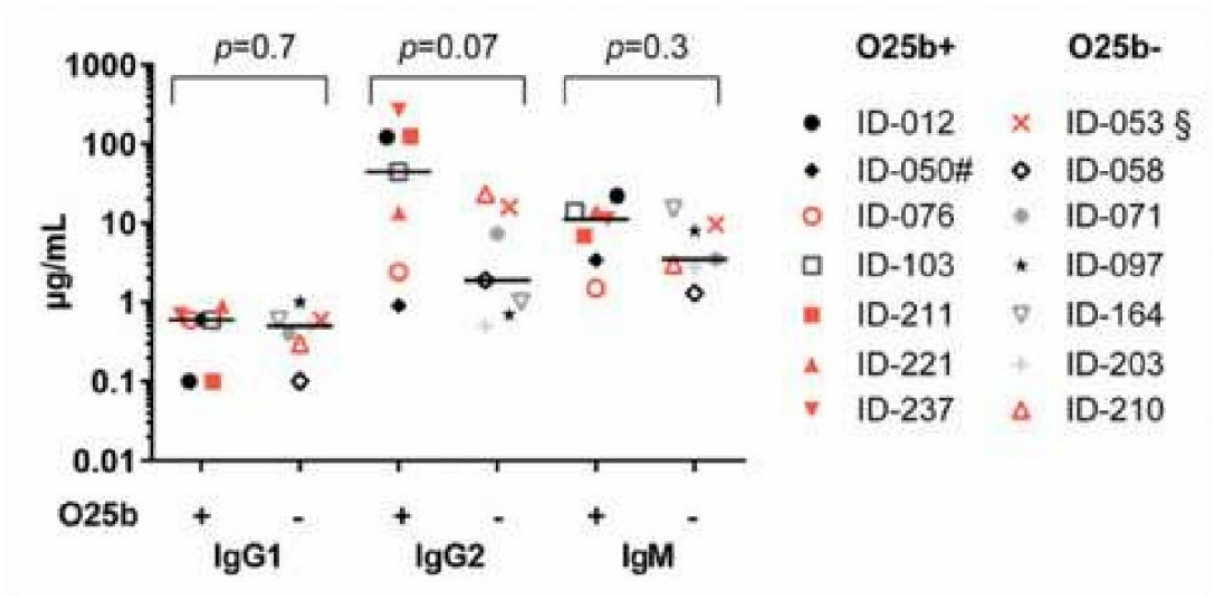
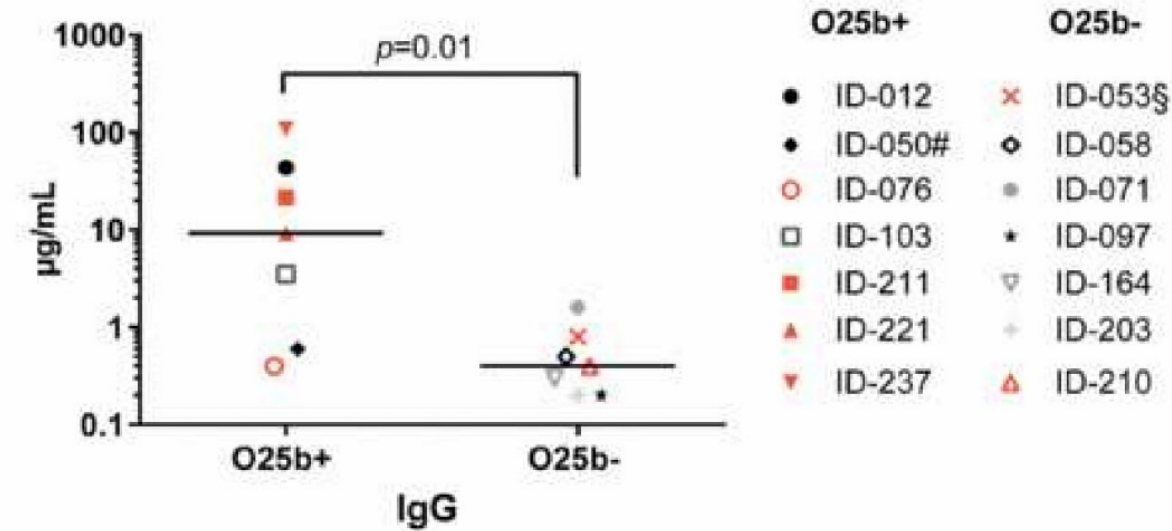


Figure 3

Figure 3

A



B

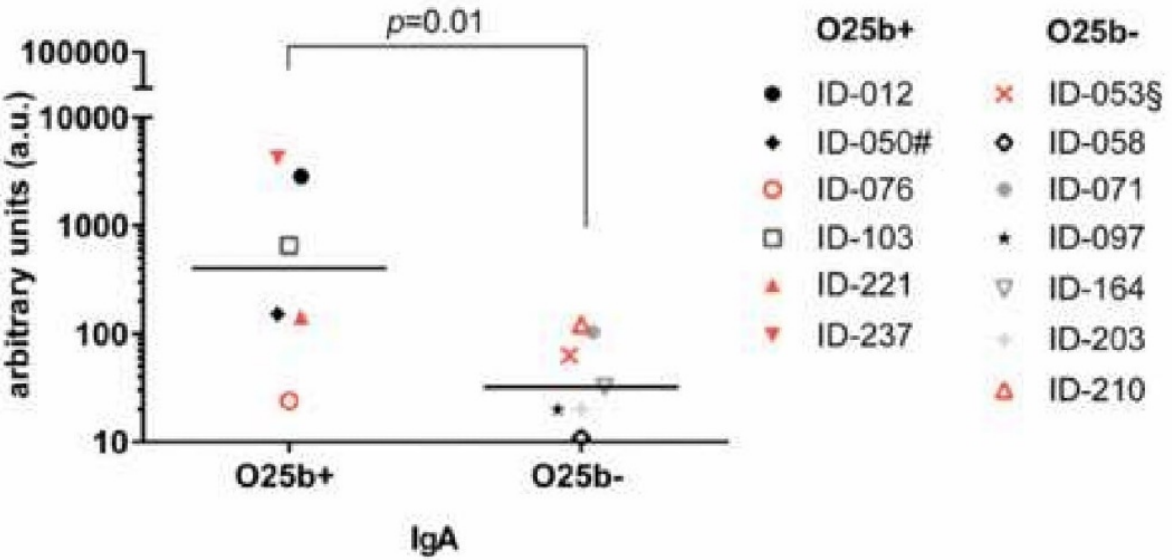


Table 1 Characterization of the *E. coli* isolates

Patient ID	Strain ID	Phylogenetic group	<i>rfb</i>_{O25b} PCR	O25b ELISA	MLST	VAP
12	LEC_001	B2	+	+	ST131	
53	LEC_004	B2	+	-	ST131	+
76	LEC_011	B2	+	+	ST131	+
103	LEC_013	B2	+	+	ST131	
211	LEC_015	B2	+	+	ST131	+
221	LEC_019	B2	+	+	ST131	+
237	LEC_021	B2	+	+	ST131	+
50	LEC_003	D	+	+	ST69	
58	LEC_007	ND	-	-	ST73	
164	LEC_014	B2	-	-	ST73	
210	LEC_017	B2	-	-	ST73	+
97	LEC_012	A	-	-	unknown*	
71	LEC_010	B2	-	-	ST1193	

*unknown – no sequence type could be assigned

Table 2 Demographics of mechanically ventilated patients with tracheal *E. coli* colonization

	ST131 n = 7		Non-ST131 n = 6		P	Odds ratio (95% CI)
Age years (range)	70	(58-82)	64	(45-79)	0.25†	
Male gender n (%)	6	(86)	3	(50)	0.27‡	6 (0.422-85.3)
BMI (range)	27.8	(17.1-39.9)	31.6	(21.5-44.7)	0.46†	
Ventilator associated pneumonia (%)	5	(71)	1	(17)	0.08‡	12.5 (0.84 – 186.4)
Mortality in hospital (%)	2	(29)	2	(33)	1‡	0.8 (0.08 – 8.48)
Medical ICU (%)	6	(86)	1	(17)	0.03‡	30 (1.47 – 612.3)
Antibiotics during ICU stay (%)	7	(100)	6	(100)		
Duration of hospitalization in days, median (range)	15	(4-22)	26	(10-48)	0.01†,*	
Ventilation days, median (range)	9	(3-14)	14	(6-21)	0.18†	
Duration of mechanical ventilation prior <i>E. coli</i> isolation, median (range)	3	(1-8)	4	(1-10)	0.49†	

† unpaired t-test, ‡ Fisher's exact test two tailed, * Excluding deceased patients

Table 3 Main clinical characteristics of patients at hospital admission

Provisional admission diagnosis	ST131	Non ST131
Respiratory failure	5	0
CAP or pneumonia	1	0
Cardiovascular	1	2
Septic shock/Bacteremia	2	1
Neurologic	3	1
Digestive/Liver	1	1
Abdominal surgery	0	2
Trauma	0	1
Other	1	1

Table 4 Distribution of virulence factors among ST131 and non-ST131 strains

Virulence Factor	ST131 n = 7	Non-ST131 n = 6	P	Odds ratio
<i>cnf</i>	0 (0)	2 (33.3)	0.19	0.12
<i>cnf1</i>	0 (0)	2 (33.3)	0.19	0.12
<i>kpsMT II</i>	5 (71.4)	5 (83.3)	1.0	0.5
<i>kpsMT III</i>	0 (0)	0 (0)		
<i>papC</i>	1 (14.3)	1 (16.7)	1.0	0.83
<i>sfa</i>	0 (0)	2 (33.3)	0.19	0.12
<i>sfaS</i>	0 (0)	0 (0)		
<i>aer (iutA)</i>	6 (85.7)	4 (66.7)	0.56	3.0
<i>aer (iucC)</i>	6 (85.7)	4 (66.7)	0.56	3
<i>hlyC</i>	1 (14.3)	2 (33.3)	0.56	0.33
<i>iroN</i>	0 (0)	1 (16.7)	0.46	0.24
hemolytic activity	0 (0)	2 (33.3)	0.19	0.12
Virulence Score (average)	3.6	4.3		

Table 5 Antibiotic non-susceptibility profile of ST131 vs non-ST131 *E. coli* isolates from tracheally colonized patients

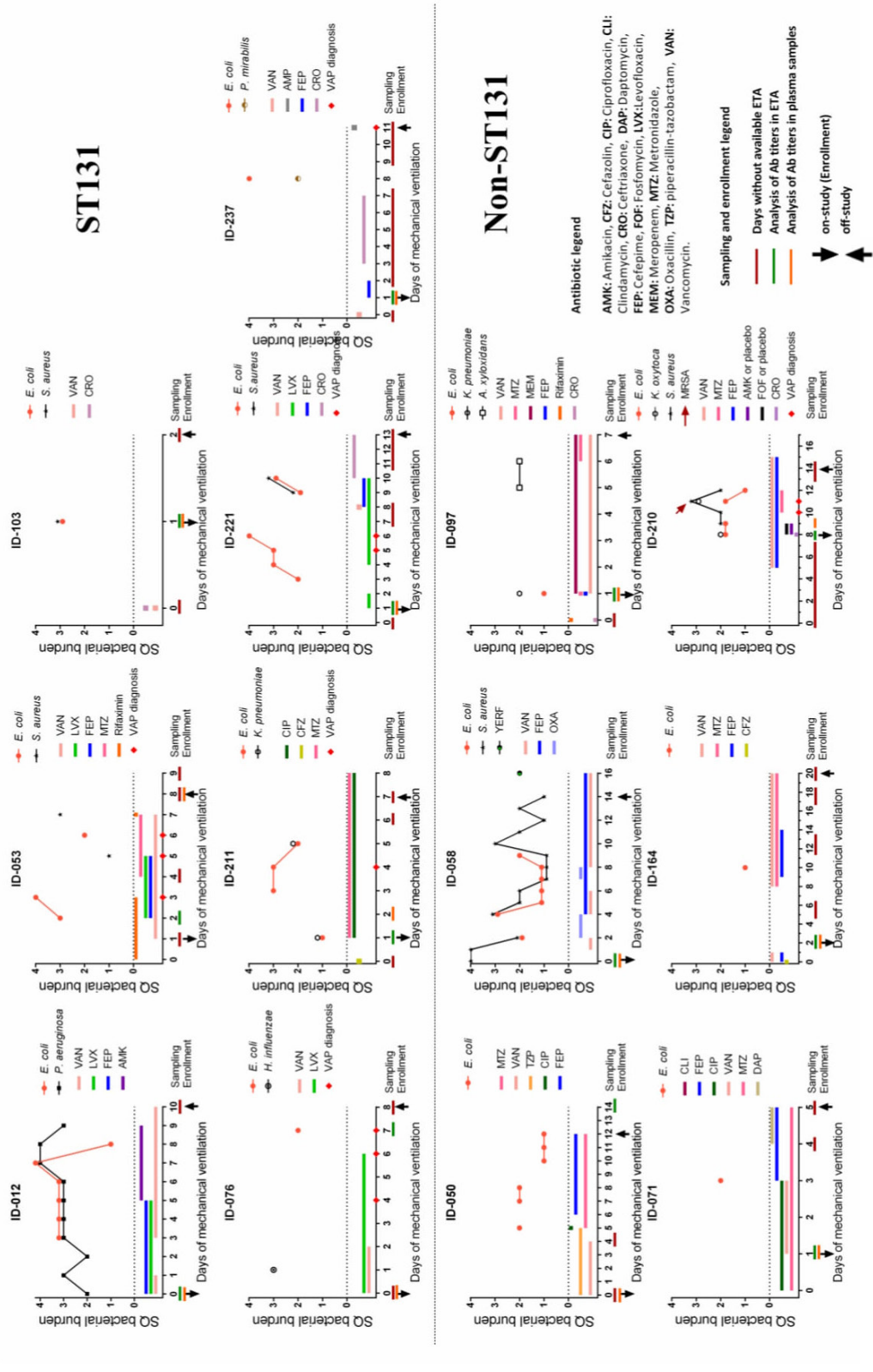
Non-susceptibility to	ST131 n = 7		Non-ST131 n = 6		P	Odds ratio
Amoxicillin Clavulanic acid	3	(42.9)	3	(50)	1	0.75
Piperacillin Tazobactam	2	(28.6)	2	(33.3)	1	0.8
Cefepime (4th)	4	(57.1)	0	(0)	0.07	16.7
Cefotaxime (3rd)	4	(57.1)	0	(0)	0.07	16.7
Ceftazidime (3rd)	4	(57.1)	0	(0)	0.07	16.7
Ceftriaxone (3rd)	4	(57.1)	0	(0)	0.07	16.7
Cefuroxime (2nd)	5	(71.4)	0	(0)	0.02	28.6
Imipenem	0	(0)	0	(0)		
Meropenem	0	(0)	0	(0)		
Gentamicin	2	(28.6)	1	(16.7)	1	2
Amikacin	1	(14.3)	0	(0)	1	3
Tobramycin	2	(28.6)	1	(16.7)	1	2
Levofloxacin	7	(100)	1	(16.7)	0.005	55
Ciprofloxacin	7	(100)	2	(33.3)	0.02	27
Tigecycline	0	(0)	0	(0)		
Trimethoprim Sulfamethoxazole	3	(42.9)	0	(0)	0.19	10.1
Colistin	0	(0)	0	(0)		

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Online Data Supplement - Figures

Supplementary Figure 1 Individual subject charts describing co-colonization, VAP development and antibiotic regimes. Semiquantitative (SQ) bacterial burden is shown in the y-axis. Days of mechanical ventilation, enrollment, antibiotic regimes and sampling days are shown in x-axis

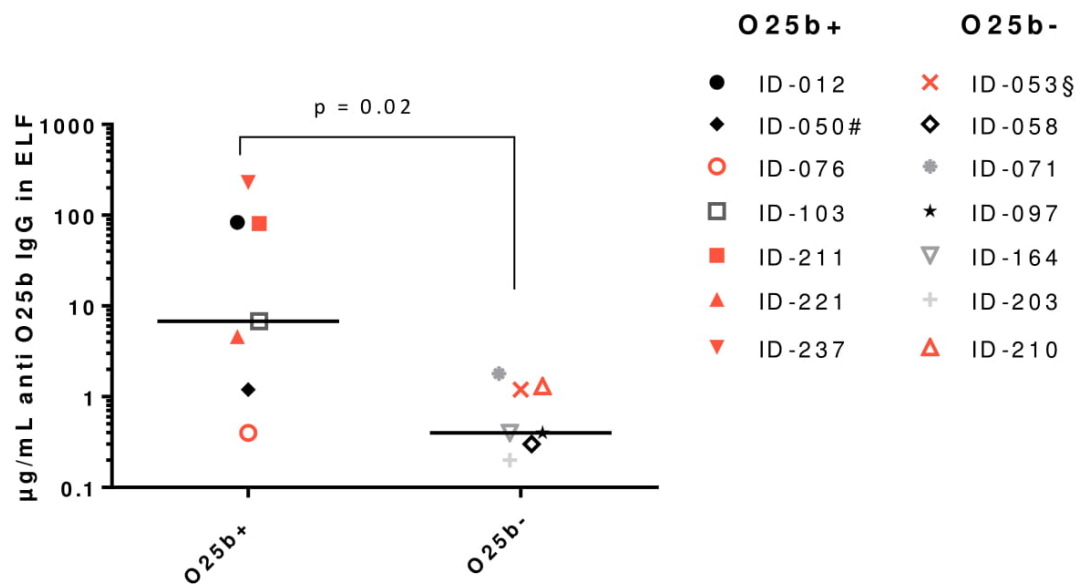


Supplementary Figure 2 O25b specific IgG titers in ELF of individual patients (normalization of IgG titers to the urea content in plasma and ETA samples determined on the same day where possible using the formula:

$$[\text{IgG ELF}] = [\text{IgG ETA}] \times \text{Ratio} [\text{Urea}]_{\text{plasma/ETA}}$$

Statistical significance is shown using the Mann-Whitney *U* test.

Only subjects 053 and 210 did not have matching urea determinations in ETA and plasma samples on the same day (sampling 2 and 1 days apart respectively). Results from subject 211 are derived from an ETA sample of a different day from Figure 3 (to matched plasma sampling on the same day)



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Online Data Supplement - Text

Supplementary methods

Determination of O25b LPS specific antibody levels in ETA and plasma samples

Anti-O25b specific antibody titers in ETA and plasma samples were measured by ELISA. ETA samples were liquefied using Sputasol (Oxoid) according to manufacturer's instructions and homogenized in bead homogenizer using 1.4 mm zirconium beads (Precellys). The sample dilution due to the addition of Sputasol was considered for quantification.

Monoclonal antibodies, specific for the O25b-antigen and sharing the same complementarity-determining region (CDR) but equipped with different Fc subclasses (IgG1, IgG2 and IgM) were used as positive controls (1). ELISA plates (ThermoFisher Scientific) were coated overnight with 100 μ L of 1 μ g/mL O25b lipopolysaccharide (LPS) purified as previously described (Szijártó et al., 2014). Blocking was performed with 2% bovine serum albumin (BSA, Biomol) in Dulbecco-phosphate buffered saline (PBS, Gibco) for 1 hour. Afterwards 50 μ L of serially diluted ETA or plasma samples in 0.1% BSA-PBS were transferred and incubated for 2 hours at room temperature. Binding of antibodies was detected using Horseradish Peroxidase (HRP)-conjugated anti-IgG, anti-IgG1, anti-IgM or using biotinylated anti-IgA or anti IgG2 antibodies followed by incubation with Streptavidin HRP (all reagents from Southern Biotech). ELISA plates were developed with Tetramethylbenzidine (TMB, ThermoFisher Scientific) stopping the reaction with 1M H₂SO₄. Anti O25b IgG, IgG2 and IgM titers were calculated based on the titration of the respective anti-O25b isotype mAb as a standard. For the specific anti-O25b IgA titers arbitrary units (a.u.) were used, where a.u. equals the dilution of the ETA sample to achieve an OD of 1.5.

Supplementary table 1 List of primers used for virulence factor determination by PCR. Table shows expected amplicon sizes, annealing temperatures and references for the respective pair of primers

Gene	Name	Secquence	Reference	bp Amplicon
<i>papC</i>	PapC-f2	GTGGCAGTATGAGTAATGACCGTTA	(Le Bouguenec et al., 1992)	200
	PapC-r2	ATATCCTTTCTGCAGGGATGCAATA		
<i>Sfa</i>	Sfa1	CTCCGGAGAACTGGGTGCATCTTAC	(Le Bouguenec et al., 1992)	410
	Sfa2	CGGAGGAGTAATTACAAACCTGGCA		
<i>SfaS</i>	SfaS-f	GTGGATACGACGATTACTGTG	(Johnson and Stell, 2000)	240
	SfaS-r	CCGCCAGCATTCCCTGTATTC		
<i>aer (iutA)</i>	aer-851f	GGCTGGACATCATGGGAACCTGG	(Johnson et al., 1997)	300
	aer-1152r	CGTCGGGAACGGGTAGAATCG		
<i>aer (iucC)</i>	aer.1	AAACCTGGTTTACGCAACTGT	(Bingen et al., 1998)	269
	aer.2	ACCCGTCTGCAAATCATGGAT		
<i>hlyC</i>	hlyCA-f	AGGTTCTTGGGCATGTATCCT	(Bingen et al., 1998)	556
	hlyCA-r	TTGCTTTGCAGACTGCAGTGT		
<i>cnf1</i>	cnf-F	CAGTGACCGGATCTCCGTTAT	(Picard et al., 2001)	230
	cnf-R	CGTGTAATTCTTCTGTACTTCC		
<i>cnf1</i>	cnf1	AAGATGGAGTTTCCTATGCAGGAG	(Yamamoto et al., 1995)	498
	cnf2	CATTCAGAGTCCTGCCCTCATTATT		
<i>iroN</i>	IRONEC-F	AAGTCAAAGCAGGGGTGCCCCG	(Johnson et al., 2000)	665
	IRONEC-R	GACGCCGACATTAAGACGCAG		
<i>traT</i>	TraT f	GGTGTGGTGCGATGAGCACAG	(Johnson and Stell, 2000)	290
	TraT r	CACGGTTCAGCCATCCCTGAG		
<i>kpsMT II</i>	kpsII f	GCGCATTTGCTGATACTGTTG	(Johnson and Stell, 2000)	272
	kpsII r	CATCCAGACGATAAGCATGAGCA		
<i>kpsMT III</i>	KpsIII f	TCCTCTTGCTACTATTCCCCCT	(Johnson and Stell, 2000)	392
	KpsIII r	AGGCGTATCCATCCCTCCTAAC		

Supplementary table 2 Virulence factor traits found in ST131 and non-ST131 *E. coli* isolates in mechanically ventilated patients. The virulence score was calculated by adding the number of positive virulence factors tested for each strain. * shows patients developing VAP

Patient ID	Strain ID	MLST	<i>cnf</i>	<i>cnf1</i>	<i>kpsMT II</i>	<i>kpsMT III</i>	<i>papC</i>	<i>Sfa</i>	<i>SfaS</i>	<i>aer (iutA)</i>	<i>aer (iucC)</i>	<i>iroN</i>	<i>TraT</i>	<i>hlyC</i>	hemolytic activity	VF score
ID-012	LEC_001	ST131	-	-	+	-	-	-	-	+	+	-	+	-	-	4
ID-053*	LEC_004	ST131	-	-	-	-	-	-	-	+	+	-	+	-	-	3
ID-076*	LEC_011	ST131	-	-	+	-	+	-	-	+	+	-	+	+	+/-	6
ID-103	LEC_013	ST131	-	-	+	-	-	-	-	+	+	-	+	-	-	4
ID-211*	LEC_015	ST131	-	-	-	-	-	-	-	-	-	-	+	-	-	1
ID-221*	LEC_019	ST131	-	-	+	-	-	-	-	+	+	-	-	-	-	3
ID-237*	LEC_021	ST131	-	-	+	-	-	-	-	+	+	-	+	-	-	4
ID-050	LEC_003	ST69	-	-	+	-	-	-	-	+	+	-	+	-	-	4
ID-058	LEC_007	ST73	+	+	+	-	+	+	-	+	+	+	+	+	+	10
ID-164	LEC_014	ST73	+	+	+	-	-	+	-	-	-	-	-	+	+	5
ID-210*	LEC_017	ST73	-	-	+	-	-	-	-	+	+	-	+	-	-	4
ID-097	LEC_012	Unkno wn	-	-	-	-	-	-	-	-	-	-	-	-	-	0
ID-071	LEC_010	ST1193	-	-	+	-	-	-	-	+	+	-	-	-	-	3

Supplementary table 3 Antibiotic susceptibility of *E. coli* strains isolated from ETA of mechanically ventilated patients. Isolates were subject to E-test. Table shows resistance (R), sensitivity (S) or intermediate category (I) of each isolate and the number of antimicrobial classes they are resistant to (median and average). Resistance to an antimicrobial class defined by resistance to at least one antibiotic of the class. * shows patients developing VAP

Antibiotic susceptibility																					
Patient ID	Antibiotic group	Penicillins		Cephalosporins					Carbapenems		Aminoglycosides			Fluoroquinolones		Glycylcyclines Tetracyclines	Folate pathway inhibitors	Polymyxin	Resistant to # of antimicrobial classes		
		Amoxicillin/Clav. Acid	Piperacillin/Tazobactam	Cefepime (4th)	Cefotaxime (3rd)	Ceftazidime (3rd)	Ceftriaxone (3rd)	Cefuroxime (2nd)	Imipenem	Meropenem	Gentamicin	Amikacin	Tobramycin	Levofloxacin	Ciprofloxacin				# of antibiotic classes	Median	Average
ID-012	LEC_001	R	R	R	R	R	R	R	S	S	S	S	R	R	S	S	S	S	3		
ID-053*	LEC_004	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S	S	1		
ID-76 *	LEC_011	S	S	R	R	R	R	R	S	S	R	S	R	R	S	R	R	S	4		
ID-103	LEC_013	R	I	R	R	R	R	R	S	S	R	I	R	R	S	R	R	S	5	3,0	
ID-211*	LEC_015	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	2	2,7	
ID-221*	LEC_019	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	1		
ID-237*	LEC_021	S	S	I	R	I	R	R	S	S	I	S	S	R	R	S	R	S	3		
ID-050	LEC_003	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	1		
ID-58	LEC_007	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0		
ID-71	LEC_010	R	R	S	S	S	S	S	S	S	R	S	R	R	S	S	S	S	3	0,5	
ID-97	LEC_012	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	0	0,8	
ID-164	LEC_014	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	1		
ID-210*	LEC_017	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0		

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CONCLUDING DISCUSSION

In medical history, the use of antibiotics allowed a dramatic improvement in the treatment of infectious disease, the main causes of death in the pre-antibiotic era. However, the continuous emergence of antibiotic resistance poses a serious threat to our health care system. In particular, the pipeline of novel antibiotics against Gram-negative bacteria is “drier” than that against Gram-positive ones and no new classes of Gram-negative antibiotics have been developed (Domalaon et al., 2018).

The dissertation provides novel insights into the host-pathogen interaction of *Escherichia coli* ST131 clonal complex with the human plasma/serum components. It sheds light on the role of the complement system in killing this clone, and on the humoral response of colonized and/or infected patients. In addition, the dissertation offers new data regarding the prevalence of ST131 in ventilated patients, and the progression of ventilator-associated pneumonia.

The *E. coli* strain 81009 has been used in all experiments carried out at Arsanis Biosciences GmbH, to investigate the ST131 clonal group. This strain was isolated in the United Arab Emirates from a male patient with UTI and used afterwards to generate O25b O-antigen specific monoclonal antibodies. Due to the importance of strain 81009, and to better characterize it, we sequenced its genome with both PacBio and Illumina methods, to obtain high-quality sequence that allowed *de novo* genome assembly. 81009 has a genome of 5,012,084 bp and a plasmid of 135,720 bp. It carries numerous antibiotic resistance and virulence factor genes. Interestingly, we identified a significant number of toxin-antitoxin systems such as CcdBA, and Kid/Kis and Phd/Doc, which previously has not been described in *E. coli*. Moreover, we found that 81009 belongs to the recently described bla^{CTM-X-27} group and our publication described the first deposited fully-assembled genome for this C1-H30R subclone. Because of the abundance of *E. coli* genome sequences present in the public databases, we could compare our strain to other members of the ST131 and non-ST131 sequence types. It was striking to note the homology among the members of this clonal group. In particular, another fully-assembled genome, *E. coli* H105 (Ghosh et al., 2017) showed almost 100% homology for both the chromosome and the plasmid.

As mentioned before, passive immunization represents an option to treat or prevent bacterial infections, as alternative or adjunct to antibiotics. Antibodies exert their main function in the blood, and from there they can extravasate into other tissues (Murphy et al., 2008). In the blood they can protect the host from systemic infections such as bacteraemia and sepsis, therefore it is important to characterize the activity of an antibody in the serum.

In my project I aimed to clarify the role of complement in the serum bactericidal activity induced by an anti-O25b mAb. In preliminary experiments we tested the role of the various complement pathways, both by depleting single complement factors from sera or by inhibiting the factors with specific antibodies (mAbs or polyclonal sera). However, it was difficult to conclude about the role of single pathways and great heterogeneity among donors was observed. Also, one of the biggest problems was the presence of pre-existing antibodies recognizing the strain of interest. As a first attempt, we treated the serum with anti- κ/λ -light chain resin, in order to obtain a complement source free of pre-existing antibodies. However, the treatment severely impaired all complement pathways tested, as C1q and MBL2 were retained by the resin. Similar findings were published by Brookes et al. (2013) when using Protein A resin.

Given the great complexity of the serum, we thought that a bottom-up approach was needed. Instead of dissecting the role of the complement by removing or inhibiting single proteins, reconstitution of the pathways from individual components was found to be attractive, offering an *in vitro* system free of confounding factors. Schreiber et al. (1979) reported the successful reconstitution of the alternative pathway from purified complement components. Although the partial reconstitution of the classical pathway was reported in several papers, no successful reconstitution of the whole classical pathway (from C1 to C9) was ever reported.

As a first attempt, we mixed all the proteins from C1 to C9. Although we observed a MAC assembly activity, it was weak and unstable. Moreover, C4 and then C3 were promptly cleaved, even without activators, leading to the consumption of the downstream factors. In a series of experiments, we found that the C1 inhibitor (C1-INH), a serpin-type protease inhibitor, was needed to stabilize the classical pathway components *in vitro* and to prevent autoactivation and subsequent consumption of factors. While similar role of C1-INH in humans was already reported (C1-INH deficiencies), this was the first report to show that it was indeed needed for the functionality of the whole pathway. Furthermore, we demonstrated, that even a ten-fold excess of C1-INH did not block the specific activation of the classical pathway induced by immune complexes (antibody-antigen), and thus we confirmed previous findings (Ziccardi, 1982). This method allowed us to combine the *in vitro* reconstituted classical and alternative pathways. The successful reconstitution of the classical pathway was a significant achievement, which enabled us to identify the minimal requirement for maximum bactericidal activity. We targeted the strain 81009 with an anti-O25b mAb using *in vitro* reconstituted complement, to dissect the role of the complement system in the bactericidal activity induced by the mAb

A1124 (IgG₁ sub-class, specific for O25b). We found that the alternative pathway alone was not able to induce any bactericidal activity, while the classical pathway (in combination with the specific mAb) resulted in bacterial killing. When both pathways were combined, the killing activity was further increased, achieving the same bactericidal activity found in normal human serum. These results suggest that in case of ST131, both the alternative and classical pathways are needed for the maximal serum bactericidal activity. Interestingly, our finding also suggests that other bactericidal factors such as lysozyme or lactoferrin are not contributing significantly to the bactericidal activity, however we cannot exclude that in certain infections or specific patient populations they play an important role.

Additionally, we tested the bactericidal activity induced by a human polyclonal immunoglobulin preparation against the laboratory prototype *E. coli* strain, MG1655. In this case, the classical pathway alone completely killed all bacteria, in agreement with the fact that MG1655, a “rough” strain, not expressing LPS O-antigen, is more sensitive to the complement action than “smooth” strains (expressing O-antigens), such as the clinical isolate 81009.

This is the first report of the successful reconstitution of the classical pathway alone and in combination with the alternative pathway. The method offers an unprecedented level of reproducibility and customization. The protocol can be modified in order to change the concentration of single factors, to study mutated versions of its components, or to supplement with other proteins, to study their effects. Moreover, the *in vitro* reconstitution offers the possibility of identifying the minimal conditions needed to achieve bactericidal activity. In our case, the method allowed us to identify the complement components required to achieve maximum mAb-dependent bactericidal activity.

LPS is an important virulence factor and one of the main surface-exposed antigens of Gram-negative bacteria, and it induces inflammation through TLR4 signalling. Thus, the modification of the lipid A is a common strategy by several pathogenic Gram-negative bacteria to evade host immunity (Maeshima and Fernandez, 2013; Tan and Kagan, 2014). Given its importance, the LPS is also an important drug target, aiming to kill the bacterium or to halt the inflammation and sepsis. Among the drugs targeting LPS, colistin (or polymyxin E) is considered the best known, and used as a last resort antibiotic to treat infections caused by MDR bacteria. It binds the lipid A, inducing damage to the bacterial membrane and blocking at the same time the TLR4 induction. However, the efficacy of colistin is undermined by the emergence and spreading of the *mcr-1* gene, which confers colistin resistance.

Despite the acknowledged importance of Mcr-1, it was unclear how this resistance factor impacts the virulence traits, such as serum survival, macrophage uptake, induction of

TLR4 signalling, of the strains carrying this gene. To address these questions, we transformed our prototype ST131 strain with a plasmid expressing *mcr-1*. Since the gene from the endogenous promoter appeared to be toxic, as demonstrated also by Yang et al. (2017), we cloned *mcr-1* under the control of arabinose-inducible promoter. We did not observe any change in TLR4 signaling, macrophage uptake, or serum survival of Mcr-1 expressing versus control (mock transformed) strains. As expected, the endotoxin neutralization of colistin was impaired in the case of the LPS extracted from the Mcr-1 expressing strain. However, compared to colistin, the activity of the anti-O25b mAb against the Mcr-1 modified LPS was unchanged. We observed no change in serum resistance of the *mcr-1* expressing strain. These observations were interesting, and highlight differences compared to other Gram-negative bacteria, like *Neisseria gonorrhoeae*. Indeed, in *N. gonorrhoeae*, the addition of phosphoethanolamine in the lipid A was reported to confer serum resistance and TLR4 hyperresponsiveness (Lewis et al., 2009). The *in vitro* findings were confirmed in a mouse endotoxemia model. Mice were first sensitized to LPS with galactosamine (Galanos et al., 1979), treated prophylactically with anti-O25b mAb, and then challenged with purified LPS modified by Mcr-1. The anti-O25b mAb protected the animals from the Mcr-1-modified LPS induced shock, the same way as against unmodified LPS challenge (Guachalla et al., 2017).. The results suggest that despite the modification in the lipid A due to Mcr-1, the LPS micelle formation and the mode of action of the anti-O25b is preserved. Therefore, we concluded that despite the resistance to colistin, *mcr-1*-carrying ST131 strains can still be successfully targeted by the anti-O25b mAb.

As described in the previous paragraphs, and suggested by previous results published by Arsanis (Guachalla et al., 2017; Szijártó et al., 2014, 2015), the use of the anti-O25b mAb might be beneficial in a population suffering from infections due to ST131. However, a better understanding of the disease progression in ST131-infected patients and the natural immune response provoked by the colonization and infection is needed. We therefore investigated this in the context of a translational study involving mechanically ventilated patients. While the presence of *E. coli* in VAP is widely reported, no data about the importance and prevalence of *E. coli* ST131 in VAP have been reported. Although some studies reported ST131 among VAP isolates, no data regarding incidence, virulence factors expression and antibiotic resistance have been provided (Dufour et al., 2016; Szijártó et al., 2012). We aimed to fill this gap by looking at a large cohort of mechanically ventilated patients enrolled in a prospective observational study. The bacterial strain collection was composed of clinical isolates from the ETA, serum samples from the corresponding patients and in addition, clinical

information was provided. This comprehensive analysis allowed us to link the bacterial characteristics and the disease progression in individual patients.

As expected from a VAP study performed in the USA, we observed a low incidence of *E. coli* compared to other bacterial species colonizing the trachea of ventilated patients. Of all *E. coli* strains, the ST types were limited to a few. The ST131 was the most prevalent, while we retrieved three ST73, one of ST69, and ST1193. Although ST131 was the predominant clone, the isolates did not carry more virulence factors than those belonging to non-ST131 types. Interestingly, all patients positive for ST131, received at least one antibiotic not effective against the carried strain, especially fluoroquinolones, while it was the case only in two out of six patients in the non-ST131 group. Moreover, almost all patients colonized with ST131 developed VAP, while it happened only to one patient colonized with non-ST131 strains. However, we did not observe any difference in the antibiotic resistance pattern among *E. coli* isolates from patients who developed VAP versus those who did not. Thus, the antibiotic resistance of ST131 might contribute to its dominance among colonizing strains, nevertheless it does not appear to be the decisive factor driving progression to VAP. Patients colonized with ST131 mounted an antibody response against the O25b antigen, but the antibodies appeared to be restricted to the IgG₂ isotype. This isotype is typically induced by carbohydrate antigens, and has a weak ability to activate the complement. Moreover also binds weaker the FcγRs (Bruhns et al., 2009), thereby it does not induce a significant phagocytosis, compared to other IgG isotypes (Murphy et al., 2008). In a previous Arsanis publication (Guachalla et al., 2017), we showed that the anti-O25b mAb with IgG₁ isotype alone was not able to stimulate phagocytosis of ST131 bacteria. However, in the presence of complement, the mAb induced a strong opsonization and phagocytosis. It is possible that high concentration of IgG₂ type O-antigen specific antibodies in the serum may protect the bacterium from being opsonized by O-antigen specific IgG₁ or IgG₃ antibodies. This hypothesis has been confirmed by the recent paper from Coggon et al.(2018), who clearly showed the pathogen protective effect of high concentration of IgG₂ antibodies by inhibiting serum bactericidal activity. .

We showed that ST131 was an effective colonizer despite standard antibiotic treatment, and ST131-colonized patients frequently developed VAP, despite the presence of specific antibodies. However, we do not know whether the colonizing strains were hospital- or community-acquired. Since the ST131 clone is a good colonizer and frequently isolated also from healthy subjects, it can stably reside in reservoirs, such as the gut, without causing any illnesses. However, upon antibiotic treatment, it may overgrow other bacterial species and

clonal lineages. This might explain the predominance of this clonal lineage in VAP over the other ST types, in spite of no greater presence of virulence factors.

Antibodies extravasate in the lungs, and this process is enhanced in presence of inflammation (Wang-Lin and Balthasar, 2018). Furthermore, the presence of the complement in the lung (Bolger et al., 2007), could theoretically induce the clearance of the bacteria through both direct bactericidal activity and enhanced phagocytosis. Accordingly, passive immunization with Arsanis' anti-O25b mAb might be a treatment option in case of lower airway infection, such as VAP, by inducing the clearance of the bacteria from the lung and counter-acting the pre-existing IgG2 antibodies. However, this hypothesis needs to be tested in more *in vivo* and *in vitro* models, given the complexity and several factors in play.

Although the data presented in this study are novel, they have several limitations. The small size of the patient group, the lack of more detailed information, the short time of observation and the fact that it is a monocentric study, prevented us to draw more general conclusions. Nevertheless, the anti-O25b mAb provides a fast, easy and accurate way to identify the presence of ST131 (Szijártó et al., 2014), which may help the health-care providers to adapt antibiotic therapy, and identify patients at high risk for developing VAP.

Taken together, the results I present in this dissertation, show that treatment with an IgG₁-type anti-O25b mAb offers a new strategy against MDR *E. coli* ST131, even when it expresses the highly concerning antibiotic resistance genes, *mcr-1*. The activity of the classical and alternative pathways of the complement are required and sufficient to induce full killing of the bacteria by this mAb, as shown by the *in vitro* reconstituted complement. Moreover, with my work I contributed to the understanding of the complex host-pathogen interaction in human serum and in the context of VAP, where the ST131 is suggested to be the dominant clonal lineage of *E. coli*. Given the expression of the O25b antigen by the multi-drug resistant ST131 lineage, anti-O25b mAbs can be used to identify and target this clonal group.

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