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Evolutionary trajectories of *Klebsiella pneumoniae*

From experimental biofilm evolution to a hospital outbreak

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Abstract

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Bacterial evolution is closely intertwined with our lives. As their hosts, we shape how bacteria evolve by imposing numerous selective pressures during the time bacteria spend in our bodies. As a result, they adapt in various ways to colonize us or infect us better. In this thesis, I present studies aimed to expand the knowledge on the pathoadaptive changes in *Klebsiella pneumonia*e, which is a bacterial pathogen of critical importance worldwide.

In Paper I, we present a new 3D-printed device for growing and studying surface-attached bacterial biofilms. The special aim was to increase the ease of use and versatility, and we have used this biofilm device to screen for biofilm capacity, perform experimental evolution and fundamental biofilm analysis in subsequent studies.

In Paper II, we study within-host evolution by analyzing 110 isolates originating from the same multidrug-resistant *K. pneumoniae* clone that caused an outbreak at Uppsala University Hospital between 2005 and 2010. We whole-genome sequenced these isolates and phenotypically characterized them to show that the clone has undergone extensive changes in individual patients, leading to increased biofilm formation capacity, attenuation of systemic virulence, and altered colonization potential.

In Paper III, we exploit an experimental evolution approach to decipher evolutionary trajectories towards increased biofilm formation. We show how fast this trait can be acquired in different *K. pneumoniae* strains by a strong convergent evolution, mostly targeting genes involved in capsule, fimbriae, and c-di-GMP-related regulatory pathways. Importantly, this genetic parallelism extends beyond *in vitro* observations as we find an extensive overlap with clinical outbreak isolates that carry signatures from within-host evolution.

The experimental evolution experiments revealed interesting genetic changes not only in the known structures or pathways but also in completely novel factors. In Paper IV, we explore a previously uncharacterized T6SS effector that is involved in biofilm formation in *K. pneumoniae* and strongly enhances this phenotype upon acquiring a single and specific point mutation. We demonstrate that the toxin acts as a DNase and that this mutation results in changes at multiple levels, including protein stability, toxicity, and transcriptional profiles, which collectively lead to the formation of biofilms.

Keywords: Klebsiella pneumoniae, evolution, experimental evolution, biofilms, infection, bacterial pathogens

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“I almost wish I hadn’t gone down that rabbit hole – and yet – and yet – it’s rather curious, you know, this sort of life!”
Alice in Wonderland

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To each and every one of You refusing to give up

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Zaborskytė, G**., Wistrand-Yuen, E., Hjort, K., Andersson, DI and Sandegren, L. 2022. Modular 3D-Printed Peg Biofilm Device for Flexible Setup of Surface-Related Biofilm Studies. Front. Cell. Infect. Microbiol. 11:802303.

II. **Zaborskytė, G**., Hjort, K., Tängström, G., Lytsy, B., Sandegren, L. Convergent within-host evolution of a multi-resistant *Klebsiella pneumoniae* clone during a 5-year hospital outbreak. *Manuscript*

III. **Zaborskytė, G.**, Coelho, P., Wrande, M., Sandegren, L. Rapid evolution of increased biofilm formation in *Klebsiella pneumoniae* via changes in capsule, fimbriae and c-di-GMP signaling. *Manuscript*

IV. **Zaborskytė, G**.[⊗], Sigurlásdóttir, S.[⊗], Dwane, R., Kjellin, J., Sandegren, L., Koskiniemi, S. A type VI secretion effector regulates biofilm formation in *Klebsiella pneumoniae*. *Manuscript*

[⊗] These authors contributed equally.
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Other papers by the author not included in this thesis:
Zaborskyte G, Andersen JB, Kragh KN, Ciofu O. 2017. Real-time monitoring of *nfxB* mutant occurrence and dynamics in *Pseudomonas aeruginosa* biofilm exposed to subinhibitory concentrations of ciprofloxacin. Antimicrob Agents Chemother 61:e02292-16.

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Contents

Abbreviations

c-di-GMP	bis-(3′-5′)-cyclic dimeric guanosine monophosphate
CAUTI	catheter-associated urinary tract infection
CF	cystic fibrosis
cKp	classical (opportunistic) *K. pneumoniae*
CI	competitive index
CPS	capsular polysaccharides
CV	crystal violet
DGC	diguanylate cyclase
ECM	extracellular matrix
ESBL	extended-spectrum β-lactamase
GI	gastrointestinal
hvKp	hypervirulent *K. pneumoniae*
LC-MS	liquid-chromatography mass spectrometry
MDR	multi-drug resistant
PDE	phosphodiesterase
PNAG	poly-β-1,6-N-acetyl-D-glucosamine
SEM	scanning electron microscopy
T6SS	type VI secretion system
TEM	transmission electron microscopy
Und-P	undecaprenyl phosphate
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection

Introduction

> "The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music." Lewis Thomas, *The Lives of a Cell,* 1974

Like every living being, bacteria replicate and introduce mistakes in their DNA, creating opportunities for evolutionary adaptation. Short generation times and large population sizes mean that adaptation can happen fast – sometimes in a matter of days. Importantly, bacterial evolution does not occur in isolation, as many of them interact with us both as pathogens and commensals, meaning that we as hosts shape their evolution, but also are directly affected by it. Thus, investigation of evolutionary trajectories in bacteria not only sheds light on universal evolutionary processes and contributes to general knowledge, but can also provide insights in how to maximize the positive interactions with the benign microbiota and fight the pathogens. Although other forces, such as horizontal exchange of genetic material, play essential roles in bacterial evolution at large, mutations are inherent to adaptative bacterial evolution. The role of genetic variation, in many cases in the form of a single point mutation, and mutation-driven adaptive evolution is the central theme throughout the work presented here. In this thesis, I explore *Klebsiella pneumoniae* and the evolutionary trajectories this bacterium undergoes both in a clinical setting and during more controlled experimental evolution, and I hope you find it as fascinating as I do.

Pathoadaptive bacterial evolution

Within-host evolution

From the point of view of bacteria, life inside a human host is a never-ending obstacle course with constant immune attacks, varying nutrient availability and diverse tissue-specific conditions. Yet, bacteria manage to survive in the human body, either as harmless colonizers (commensals) that do not hurt us and can even be beneficial, or as pathogens causing infections. While this is largely due to an ever-ongoing host-pathogen evolution on a macroscale, fast growth or prolonged chronic/recurrent infection means that mutational diversity, the inherent force driving adaptive evolution, arises even during the

course of life in a single host (Lieberman et al., 2013; Jousset et al., 2018; Lee et al., 2019; Wylie et al., 2019; Aihara et al., 2021; Hjort et al., 2022; Yang et al., 2022). The spectrum of conditions at the site of colonization or infection inevitably results in the selection of certain genetic variants with an advantage (increased fitness) in specific conditions (Figure 1).

For opportunistic pathogens like *K. pneumoniae*, there are several ways to support growth, and thus mutation supply, within the host. One of them is asymptomatic colonization, which precedes the infection, thus extending the period that the pathogen spends inside the host. During this rather mysterious bacterial-host interaction, pathogens can silently live in the gut, on the skin, or on mucosal surfaces without any signs of their presence, unless specifically sought. During colonization, there are ample opportunities for the bacterium to spread to other hosts via direct contact, droplet transmission, or faecal contamination. Colonization in the gut by CTX-M-15 producing *K. pneumoniae* has been shown to last more than 12 months with a shorter average colonization in adults (2.5 months) and longer in infants, especially if they received antibiotics and were delivered by caesarean section (Löhr et al., 2013). However, it can span even longer periods of time. For instance, a patient carried the same strain of carbapenem-resistant *K. pneumoniae* for 4.5 years after being colonized from a contaminated endoscope (Jousset et al., 2018). The common route of infection for *K. pneumoniae* is from colonization of the gastrointestinal tract to colonization of the urinary tract (Martin et al., 2016b; Gorrie et al., 2017). The environment of the urinary tract differs significantly from that of the intestines. While rich sugar-based carbon sources and competition with the endogenous microbiota prevail in the intestines, the urinary tract is typically a nutrient-poor environment with limited iron and dominated by amino acids and peptides, scarce bacterial competition, and fierce host defense (Alteri and Mobley, 2012, 2021). Thus, it is not difficult to imagine that different genetic variants would have an advantage in these two different niches. Adaptive mutations creating variants that are better at causing infections have been shown to arise both during colonization and infection in numerous bacterial pathogens (Lysenko et al., 2010; Young et al., 2017; Yang et al., 2022). For example, *Staphylococcus aureus* acquired mutations in genes related to pathogenesis during nasal colonization and proceeded to cause blood and deep-tissue infections (Young et al., 2017). Similarly, *Streptococcus pneumoniae* underwent selection towards an increased propensity to cause invasive disease during colonization in the nasopharynx, where the competition with another bacterium *Haemophilus influenzae* selected for increased capsule production (Lysenko et al., 2010). *Enterococcus gallinarum* colonizing the gut was recently shown to diversify during colonization and lead to distinct lineages that either continue colonization or translocate to the liver and induce inflammation (Yang et al., 2022). The *K. pneumoniae* mentioned above, which colonized a patient for 4.5 years, eventually resulted in the patient's death due to the evolution of much better serum survival and biofilm growth,

although it restored susceptibility to colistin (Jousset et al., 2018). Our study of an outbreak at Uppsala University Hospital (**Paper II**) illustrates the propensity of a *K. pneumoniae* clone to both colonize the gastrointestinal tract (GI) and lead to infections, in many cases urinary tract infections (UTI), and how mutations leading to different phenotypes appear.

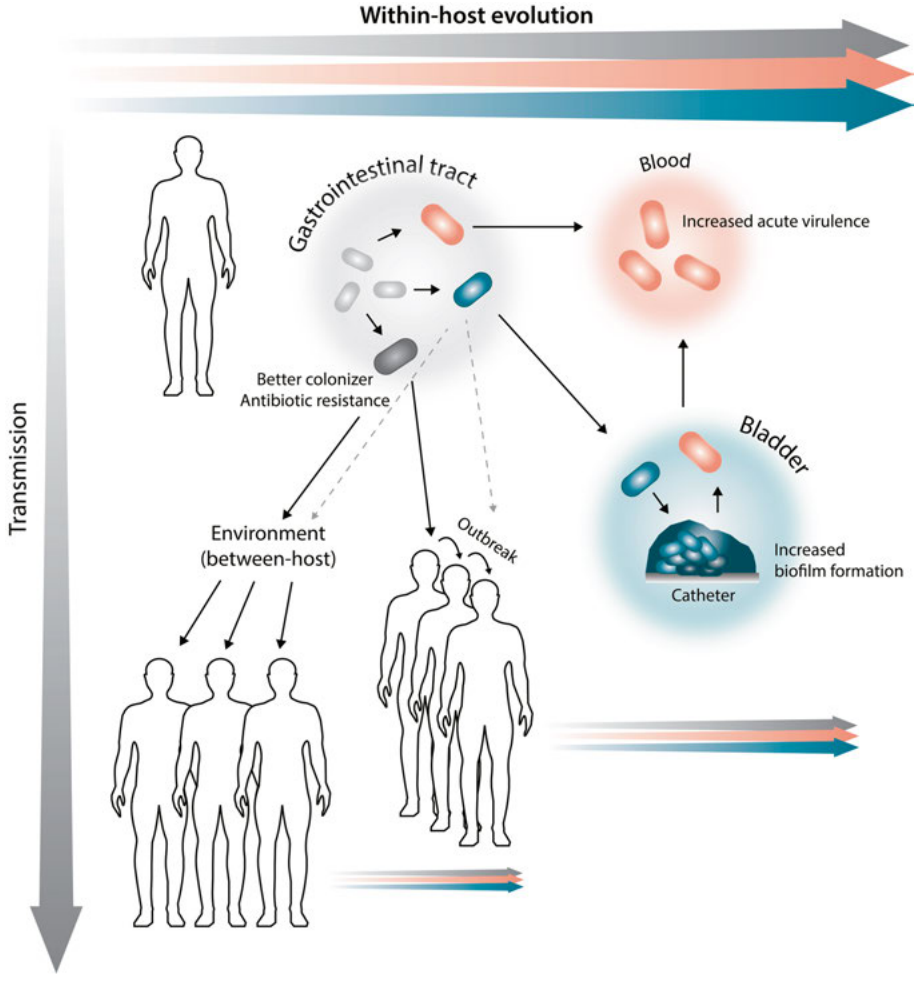

Figure 1 | Examples of possible evolutionary scenarios during *K. pneumoniae* colonization, infection and transmission.

Another factor allowing bacterial populations to live inside the host for extended periods of time is biofilm formation. Biofilms (discussed in much more detail later on) represent bacterial communities that often reside on medical devices (e.g., urinary catheters, endotracheal tubes, implants) or attached to tissues/embedded in sputum, and that are tolerant to antibiotic treatments and immune factors (Høiby et al., 2015; Percival et al., 2015; Koo et al., 2017; Ciofu et al., 2022). Therefore, by definition, they would not experience the

same host-induced bottlenecks (i.e., reductions in population size) as non-biofilm populations. Furthermore, biofilm growth due to spatial constraints results in the formation of distinct niches with their own microenvironments. From an eco-evolutionary point of view, this supports microevolution even within the biofilm, where multiple clones with beneficial mutations can be kept in the absence of direct competition between them (increased clonal interference (Gerrish and Lenski, 1998)). Collectively, this can result in increased mutational diversity in a population that is maintained for extended periods of time – a perfect combination for possible adaptations to arise. Certain well-studied biofilm-associated infections, like lung infections in cystic fibrosis (CF) patients, clearly show that adaptations within lungs of individual patients occur during these chronic infections that can span decades (Lieberman et al., 2013; Markussen et al., 2014; La Rosa et al., 2018). They undergo extensive diversification with historical imprints from various selective pressures, such as metabolic adaptations (slower growth rate, increased oxygen requirement, auxotrophies), changes in iron scavenging strategies, and antibiotic resistance, among others. In addition to adaptation within the same host niche (i.e., the site where biofilms initially are formed), mutant subpopulations with an advantage in other niches or better systemic survival can arise, and upon dispersal from the biofilm, they can reach and inhabit other sites and/or cause systemic infections. However, there is less data on similar adaptations with medical device-associated biofilms.

In addition, biofilm growth itself has been suggested to increase mutation rates (Driffield et al., 2008; Flynn et al., 2016). Mutator strains with mutations inactivating mismatch repair system genes (e.g., *mutS*) have been observed in uropathogenic *E. coli* (UPEC) and *P. aeruginosa* in CF lungs, accounting for up to 20% of all isolates in contrast to <1% rates in acutely infected patients (Oliver et al., 2000; Denamur et al., 2002; Ciofu et al., 2005). In theory, increased mutation supply gives natural selection more material to work on, thus speeding up the adaptation, as shown for experimentally evolving biofilm lineages that had increased mutation rate compared to planktonic lineages and resulted in highly diverse populations (Flynn et al., 2016). In addition, the spectrum of resistance mutations experimentally selected in wild-type and mutator backgrounds differs (Cabot et al., 2016). However, considering that most mutations are deleterious, if the mutation rate is increased too much or for too long, an overwhelming number of harmful mutations accumulate, making it hard for natural selection to sift through and keep the beneficial ones. Therefore, a mutation rate that is too high may neutralize adaptive evolution (Gerrish et al., 2013). While in the presence of strong stressors, such as antibiotic treatments, or under fluctuating conditions, a high mutation rate can be favored and lead to adaptation, hypermutators are probably not as beneficial once an adaptation has occurred or when the conditions are more stable (Van den Bergh et al., 2018). In a large scale genomic dataset on evolutionary trajectories in multiple bacterial species, high mutation rates were also associated

with the start of the infection (Gatt and Margalit, 2021), perhaps reflecting the benefit of generating as much diversity as possible in the beginning of life inside the host, although this study did not differentiate infections based on biofilm involvement.

When it comes to survival in the host, the possibilities of adaptive changes are infinite, considering the metabolic requirements, competition with other bacteria during colonization or within polymicrobial biofilms, immune system attacks, antibiotic treatments, and combinations of all of these factors. However, in order to be maintained in a longer evolutionary perspective, optimization of growth inside an individual host is not beneficial unless the bacterium can also maintain spread to additional individuals, and a recent review grouped mutations as "adapt-and-live" or "adapt-and-die" to illustrate the different fates of bacteria that acquire them (Culyba and Tyne, 2021). The possible maintenance in the population largely depends on i) chance, that is where the mutant physically ends up (e.g., gut vs blood), and ii) possible trade-offs in other environments/niches. From an evolutionary point of view, certain infections, for example, UTI and blood infections, are not the most successful route for a bacterial pathogen because they typically result in a dead-end or at least very unlikely transmission, in contrast to gut colonization where transmission is much more likely. In such cases, the "short-sighted evolution" concept could be applied (Levin and Bull, 1994), where adaptive changes specific to a certain niche are selected simply because this results in the best adaptation in the given condition. As Sokurenko et al. suggest, this is especially true for opportunistic pathogens where the "source" (i.e., colonization site) is different from the "sink" (i.e., infection site) (Sokurenko et al., 2006). Colonization essentially decouples transmission from the pathogenic process, and opportunistic pathogens can afford some subpopulations to go extinct because it can still be transmitted from the colonization site.

An adaptation selected in one niche, for example, during a UTI can be so specific that it confers a significant fitness cost in other environments. In other words, one cannot be perfect at everything; therefore, many phenotypic changes are likely accompanied by certain trade-offs (Shoval et al., 2012; Ferenci, 2016). For example, the fimbrial adhesin FimH mutation G66R with much stronger binding phenotype at low shear forces was shown to arise in an *E. coli* in a single patient, but the trade-off in other environments limited its spread in the population (Weissman et al., 2007). However, different genetic variants of FimH with intermediate improvement in binding in the urinary tract are still kept in the population despite the relative cost to gut colonization, illustrating adaptation to a pathogenic lifestyle at large (Sokurenko et al., 1998). If such a genetic variant gets transmitted to a person with severely compromised immune status and antibiotic-disrupted microbiota, a common status in hospitalized patients, the short colonization step perhaps would not hinder it from reaching the urinary tract and establishing itself there. In **Paper II**, we

show another such example where a possible adaptation to UTI in a hypermucoid isolate leads to a defect in gut colonization.

Some adaptive mutations, such as those conferring antibiotic resistance, have more or less similar probability of being selected at both the colonization site and the infection site. This can, of course, depend on the antibiotic if the pharmacokinetics/pharmacodynamics of a certain drug result in different concentrations, for example, in the bladder. But considering systemically acting antibiotics, even if the infecting population is eliminated from further transmission, mutants selected due to exposure in the gut can still be transferred to other hosts from the colonizing population, reflecting an "adapt-and-live" scenario. Although not the result of within-host evolution in its strictest sense, the evolution of globally disseminated clones of *K. pneumoniae* also points to maintenance of antibiotic resistance as "adapt-and-live" changes, for example in carbapenem-resistant ST258 (Jacobs et al., 2014) and other MDR clones in other species, such as methicillin-resistant *S. aureus* (MRSA) or vancomycin-resistant enterococci (VRE) . This in general illustrates that when it comes to nosocomial pathogens, within-host evolution is directly coupled to global dissemination of certain clones or ST groups (David et al., 2019), especially in this age of global travel.

The selection and fate of pathoadaptive changes related to virulence is more complicated. Do adapting bacteria become more or less virulent? Virulence (i.e., host damage) in general is a tricky concept in the case of opportunistic pathogens, chronic infections and biofilms. If the population in the form of a biofilm is maintained for years, but does not result in acute host damage, is it less virulent? The question is not that straightforward to answer. Attenuation in acute virulence or a shift to chronic virulence is commonly observed in different pathogens, for example, *P. aeruginosa*, *E. coli, A. baumannii, S. aureus,* and others (Didelot et al., 2016; Gatt and Margalit, 2021). We also show in **Paper II** that many outbreak isolates were attenuated in acute virulence (serum survival and *Galleria mellonella* larvae model), although increased virulence examples were also present. Similarly, the above-mentioned example of a 4.5-year colonization by *K. pneumoniae* shows the direct devastating effects of increased virulence by within-host adaptation. Various hypotheses have been proposed for how virulence can evolve as reviewed elsewhere (Alizon et al., 2009). The leading one, is the classical trade-off hypothesis that states that for a long-term host-pathogen interaction virulence cannot increase so much that it would limit transmission to other individual hosts. However, such a theory does not explain the evolution when there is no direct link between the infectious process and transmission, which is usually the case with opportunistic pathogens (Sokurenko et al., 2006; Brown et al., 2012), therefore, supporting the above-mentioned "short-sighted evolution" for virulence. The alternative theory is that virulent properties having an effect on the host is just a pure co-incidence of adapting to other environments, although variations exist in whether host factors can have some effect too (Alizon et al.,

2009). Somewhat along the same lines is a proposal that the capsule in *K. pneumoniae* evolved not because of a protection against the host's immune system, but rather as an adaptation to environmental conditions, since capsule maintenance during serial passaging depends on the nutrient status (Buffet et al., 2021) and the extreme diversity of capsule types could illustrate protection from protists and phages (Wyres et al., 2016). In line with these studies, environmental *K. pneumoniae* isolates can be equally pathogenic as those in a clinical setting (Struve and Krogfelt, 2004), and in the case of opportunistic *K. pneumoniae*, it is often not the presence or absence of certain genes, but rather the host status that determines the outcome of their interaction (Gorrie et al., 2022). However, the fact that isolates from patients show changes in features related to the pathogenic process (e.g., capsule, fimbriae), selection is clearly acting on them within-host even if their evolutionary origin might not be directly linked to the host. We and others also see that both increased capsule and capsule loss mutants of *K. pneumoniae* are common from infection isolates, in our study almost exclusively from UTIs (**Paper II**), and both are able to form biofilms (**Paper III**), suggesting that maybe they both are adaptations but at a different stage/location (Ernst et al., 2020).

Another factor that is not taken into account by classical virulence evolution theories is phenotypic plasticity, that is the ability to physiologically adapt via, for example, expression regulation without mutation (Brown et al., 2012). For instance, in many opportunistic pathogens, a certain factor is not simply present or absent in the strain, but can be precisely regulated, like type 1 fimbriae regulation by phase switching (described in more detail in the section *Fimbriae*) (Schwan, 2011). In this case, while the trait itself is not universally advantageous (UTI vs intestines), it is kept in the population to allow up- or down-regulation when needed. Similarly, *K. pneumoniae* can remodel its lipid A, part of the LPS structure, which has been shown to occur in the lungs, thus contributing to niche-dependent virulence via regulatory pathways (Llobet et al., 2015). The same applies to a majority of factors that are in one way or another involved in the infection process.

Since mutations favoring adaptation to a specific infection site (e.g., bladder, blood) are more likely to be selected at these infection sites, they are much less likely to be transmitted outside the host, representing "adapt-and-die" changes. However, if a more virulent mutant arises during colonization and does not confer a significant trade-off for survival during colonization, it can still be transmitted. In addition, while colonization itself by strict definition is not a virulence factor (i.e., inducing host damage), in the case of opportunistic pathogens, where colonization often is the intermediate step to infection, inability to colonize can directly translate to lower probability of infection. Therefore, increased colonization phenotypes that can be selected at the colonization site can represent an "adapt-and-live" pathoadaptive change.

It is important to consider that, more often than not, these distinct types of adaptive change can be combined together in the same clone or even conferred

by the same mutation. For instance, loss-of-function change in outer membrane porins can have a selective advantage both in evading antibiotic treatments but also by becoming less susceptible to immune system activation, while causing metabolic challenges to the cell due to decreased nutrient uptake. In this case, it is the combination of different selective pressures and their relative strength as well as pure chance of timing between the occurrence of the mutation and surrounding conditions, that determines whether the changes "continue to live" or are eliminated.

Studying evolution in real life

How do we find the adaptations selected in the actual patients? Within-host bacterial evolution can be studied on multiple scales and by different approaches that can be divided in terms of isolate sources (databases vs clinical data from a single patient or multiple patients), type of analysis (genomic vs phenotypic or combined), and the clonality of the pathogen of interest. The exact criteria to consider a certain change as adaptive then also depend on the approach chosen.

One of the methods is individual patient analysis, where consecutive bacterial isolates are collected at different timepoints during colonization/infection. The requirement for such an approach to be possible is that the pathogen has to stay inside the host long enough for selection of genetic variants to occur and be sampled. As discussed earlier, it can be achieved if the infection is long enough, for example during chronic or recurring infections, such as those caused by *Mycobacterium tuberculosis* (Hjort et al., 2022), *P. aeruginosa* (Markussen et al., 2014; La Rosa et al., 2018) or *Burkholderia dolosa* (Lieberman et al., 2013), or if bacteria can colonize the host like *K. pneumoniae* (Jousset et al., 2018; Lee et al., 2019; Wylie et al., 2019; Aihara et al., 2021). For example, a longitudinal study of *P. aeruginosa* from the same CF patient and multiple locations (sinus vs sputum) over the span of 32 years showed that it leads to evolution of divergent lineages (Markussen et al., 2014). In the earlier mentioned 4.5-year-long case of colonization with *K. pneumoniae*, 17 isolates were collected from the same patient, allowing researchers to pinpoint the evolution with great detail (Jousset et al., 2018). In order to be informative enough, one has to make sure that the isolate diversity reflects the actual diversity within-patient, because otherwise the link between ancestral and evolved clone/population is missing. This is not always easy to achieve with a single isolate, especially with chronic infections, where compartmentalization is common, for example, within lungs (Lieberman et al., 2013; Markussen et al., 2014; Hjort et al., 2022). Therefore, it is preferable to collect populations or multiple clones from different sites if possible. Unless coupled to a more detailed analysis of phenotypic changes, the identification of real adaptations is not always straightforward from a single patient data. Furthermore, often multiple clones of the same species infect patients

simultaneously, making it difficult to identify which genetic changes are adaptive and which ones represent allelic variation, especially in the case of *K. pneumoniae*, which is extremely diverse even within the same hospital (Holt et al., 2015; Long et al., 2017).

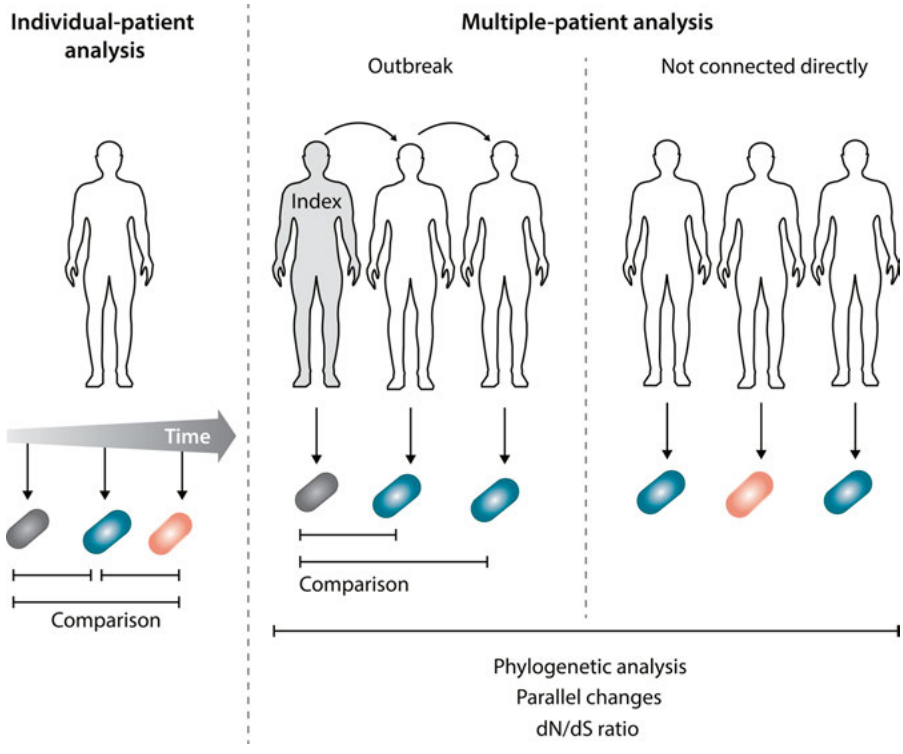

Figure 2 | Different approaches to study bacterial within-host evolution in real life.

Since opportunistic pathogens are often nosocomial, their spread from host-to-host is greatly facilitated, especially because they can silently spread undetected, and often result in outbreaks that can be short-lived or last for years (Lytsy et al., 2008; Onori et al., 2015; Gu et al., 2018; Marsh et al., 2019). In this case, the same clone or multiple related or unrelated clones can be undergoing within-host evolution in parallel. Therefore, bacterial outbreaks involving many patients can facilitate identification of truly adaptive changes by comparing multiple lineages that evolve at the same time (Lieberman et al., 2011). As with individual patient data, when related but not identical isolates infect people, extracting the truly selected mutations is even more challenging. With multiple patients, phylogenetic reconstruction is typically performed to analyze the genetic relationship between isolates. This in itself can give information about any dominant lineages ("adapt-and-live"), or more individual selection inside the host visible from lack of clear clustering ("adapt-and-die"). Classical signatures for adaptive evolution include the ratio between

nonsynonymous and synonymous changes in protein coding genes (dN/dS ratio) and signs of convergent evolution in isolates from different patients (i.e., independent evolution of similar traits) (Aguileta et al., 2009), which is not achievable with individual patient data. In simple terms, a dN/dS ratio > 1 is an indication of a positive selection pressure acting on the gene with the assumption that nonsynonymous changes are more likely to alter the phenotype, while a ratio < 1 would signal purifying selection. The absolute numbers can be affected by various parameters, such as codon bias. However, intergenic regions comprise 10-15% of genomes, and contain important regulatory elements, such as promoters, terminators, riboswitches, small RNAs and regulator binding sites, thus making it likely for selection to target them as well (Thorpe et al., 2017). It is, however, more difficult to assess, especially without any phenotypic analysis. For example, Khademi et al. described within-host adaptation by *P. aeruginosa* driven by intergenic changes (Hossein Khademi et al., 2019). Among the identified potentially adaptive changes were metal (iron, zinc) acquisition and metabolic changes (sphingolipid utilization). The authors speculated that changes in transcriptional regulation, mediated by the intergenic changes (e.g., promoters) could help adaptation in environments with multiple stressors, and that the relative contribution of coding vs non-coding evolution could depend on the environment. In the clonal outbreak presented in **Paper II**, the majority of changes were nonsynonymous mutations in protein-coding genes; however, intergenic regions were targeted as well, some of them (e.g., *fepA/fes*) – multiple times in many isolates, hinting at possible adaptation too.

Most studies analyzing evolution during outbreaks focus only on genomics and epidemiology based on the criteria described above or with more targeted searches for specific changes (Snitkin et al., 2012; Onori et al., 2015; Conlan et al., 2016). For example, Snitkin et al. analyzed an outbreak by a carbapenem-resistant *K. pneumoniae* in 18 patients, focusing primarily on reconstructing transmission networks and detecting mutations for colistin resistance (Snitkin et al., 2012). Conlan et al. focused on resistance plasmid dynamics in a smaller-scale study (Conlan et al., 2016). Sometimes, a combination of outbreak sampling and consecutive individual patient sampling is used, although this can be practically challenging. For example, Lieberman et al. combined individual patient analysis with comparison to populations from other patients to study the diversification of *B. dolosa* during long-term adaptation in CF lungs (Lieberman et al., 2013). This allowed identification of diverse selective pressures (iron availability, antibiotic treatments) within bacterial populations from each patient and compare them to those of other patients, expanding the resolution and at the same time assessing parallelism. Local outbreaks can also be a smaller illustration of global dissemination of certain clones. For instance, a study in an Italian hospital revealed the presence of the same clonal lineages as detected nationwide (Onori et al., 2015). This in general illustrates that when it comes to nosocomial pathogens, within-host evolution is directly

coupled to global dissemination of certain clones or ST groups (David et al., 2019). Consistent with the "adapt-and-live" concept for antibiotic resistance mutations, most globally disseminated clonal groups of *K. pneumoniae* are multidrug-resistant (MDR) rather than specialized for a specific type of infection. The absolute "winner" in the case of *K. pneumoniae* is ST258 (Jacobs et al., 2014; Wyres et al., 2019b), but there are examples of others, such as ST307 that has emerged in Norway (Wyres et al., 2019a). A similar approach has been applied to compare MDR and hypervirulent clones on a global scale (Bialek-Davenet et al., 2014; Wyres et al., 2019b).

In addition to outbreaks and globally disseminated clonal groups, multiple unrelated isolates can be pooled together to identify macroscale evolutionary trajectories, for example, adaptation to a specific environment. Dettman and Kassen compared 1,000 genomes of *P. aeruginosa* from CF, acute infections and environmental sources to show how CF-causing strains come from various sources but that they experience distinct convergent evolution specializing to the CF lung niche (Dettman and Kassen, 2021). A recent study analyzed data available in databases for 11 different species (*P. aeruginosa*, *M. tuberculosis*, *S. aureus*, *K. pneumoniae*, *Salmonella enterica* subs. enterica serovar *Typhimurium*, *E. coli*, *E. faecium*, *H. influenzae*, *Campylobacter jejuni*, *Clostridioides difficile* and *A. baumannii*) to compare evolutionary trajectories among them (Gatt and Margalit, 2021). The authors reported common trends such as bias towards frameshift mutations, alteration in iron and other metal uptake, cell surface structures, positive selection in transcriptional regulators and loss-of-function changes in virulence factors, thus probably leading to attenuation of acute virulence.

Genomic analysis can be a powerful predictor of adaptive evolution. However, there are a few caveats to the purely genomic approach. Large-scale genomic analysis often combines unrelated genomes from available databases, thus by default lacking any phenotypic information. For pathogens like *K. pneumoniae* exhibiting immense genetic diversity and genomic plasticity (Holt et al., 2015; Gorrie et al., 2022), identification of real adaptive changes versus simple allelic variation is a real challenge. Ultimately, it is the phenotype, and not the genetic change itself that is selected and convergence might not be visible on genomic data, thus making it hard to predict true adaptations. For instance, an 8-year-long study of *P. aeruginosa* in CF lungs identified genetically distinct trajectories that phenotypically lead to convergence via metabolic adaptation (La Rosa et al., 2018), a finding that would not have been possible without the actual experimental validation of the metabolism. In many cases, it is impossible to predict how exactly the phenotype is affected. Chen et al. identified the *fimH* adhesin gene as clearly under positive selection in UPEC, but phenotypically it was determined to be not due to an expected better binding to mannosylated receptors, but rather because of better formation of intracellular bacterial communities (IBC) (Chen et al., 2009). In addition, different combinations of mutations can affect the phenotype

differently, for example, due to epistatic effects, which are impossible to decipher purely by looking at genomes. Finally, genomic analysis is extremely sensitive to the status of annotation databases whose completion vary greatly depending on species, and can lead to functional groups that are essentially meaningless.

Sometimes additional phenotypic analysis is included in within-host evolution studies, for instance, Marsh et al. analyzed evolution of 136 related ST258 lineages over a long period of time (8 years) and performed limited phenotypic characterization in terms of biofilm formation, mucoviscosity, and iron binding (Marsh et al., 2019). In this case, the authors concluded that certain phenotypic traits coupled to genetic changes (better survival under low iron conditions due to mutations in *sufB*) could have been the reason for the maintenance of certain persistent clones. Howden et al. combined *in vivo* evolution with consecutive before and after treatment isolates from five patients with more detailed phenotypic characterization to show that decreased biofilm formation and virulence are trade-offs to vancomycin/daptomycin resistance in *S. aureus* (Howden et al., 2011). Combined genotypic and phenotypic analysis allowed pinpointing certain evolutionary events and connecting them to increased virulence and resistance to mupirocin and chlorhexidine during a clonal outbreak of community-acquired MRSA (Copin et al., 2019). In **Paper II**, we analyzed 110 colonizing and infecting isolates originating from the same ESBL-producing *K. pneumoniae* clone during a 5-year hospital outbreak. We combined whole-genome sequencing with the phenotypic characterization of various infection-related phenotypes (antibiotic resistance, serum survival, infections in the *G. mellonella* model, biofilm formation on silicone). Although we could decipher some genotype-phenotype connections, quite a few isolates have undergone extensive changes in individual patients, making it hard to disentangle exactly what mutation is responsible for a certain phenotypic change. Such an analysis in unrelated clones would be even more difficult.

From a scientific point of view, a clonal outbreak with multiple consecutive isolates from individual patients, preferably both from the colonization site and during infection, analyzed from both a genomic and phenotypic perspective, would be the most informative setup to study evolutionary within-host trajectories. More often than not, this is practically challenging both because the spread of outbreak clones can be silent for a long time and because of the vast scope of such an experimental approach. Additionally, deciding which phenotypes are the most relevant requires prior experimental data. Often, the only way to better understand within-host evolution is to try to "replay" it in a more controlled setup under specific selection conditions, as discussed in the next section.

Experimental evolution

The selective pressures that act during life inside a host are multifactorial and, in many cases, too complex for us to be able to decipher the exact reason why certain genetic variants are selected. To simplify the analysis, one can turn to a more controlled experimental evolution approach, where bacteria adapt to specific challenges imposed by the experimenter. Due to their short generation times and large populations, bacteria make attractive objects in real-time evolutionary studies as opposed to multicellular organisms. Contrary to gene inactivation studies, the experimental evolution avoids the bias of loss-of-function mutations, instead allowing us to observe what natural genetic variants would be selected under given conditions (Elena and Lenski, 2003). In addition, more targeted gene disruption studies often require prior knowledge or an educated guess about what might be important for a certain phenotype, while experimental evolution can reveal completely new factors.

The classical procedure to experimentally evolve bacterial populations involves serial transfer/passaging to allow continuous growth of the bacterial population, and thus a spontaneous mutation supply. An alternative is to grow cultures in a chemostat, which is practically more challenging. The longest still ongoing bacterial evolution experiment was initiated by Richard Lenski in 1988 with 12 lineages of *E. coli* growing in a simple setup of shaking cultures with minimal medium supplemented with glucose and diluted 100-fold each day to continue growing (https://the-ltee.org/). The number of generations these cultures have undergone would correspond to more than 1,000,000 years from a human perspective. Even in this simple setup, the researchers were able to get insight into general evolutionary questions concerning, for example, fitness changes in constant environments or convergent versus divergent trajectories of adaptation (Lenski et al., 1991; Gerrish and Lenski, 1998; Barrick et al., 2009).

Experimental evolution has become a popular approach in microbiology, especially after the onset of whole-genome sequencing that has revolutionized the possibility to take snapshots of genetic diversity in evolving populations and elucidate the exact genetic changes driving evolution (Van den Bergh et al., 2018). Commonly, experimental evolution is followed by competition experiments, where the evolved mutants are competed together with the ancestral strain to assess their relative fitness under certain conditions (e.g., growth rate, biofilm production, colonization, and virulence). The identified fitness costs or gains can then be assigned to specific genetic changes. As mentioned above, improvement of one phenotype often comes at the cost of worsening another one (Shoval et al., 2012; Ferenci, 2016). In an attempt to "fix" the trade-off, continued natural selection can lead to the appearance of compensatory changes – compensatory evolution. The mutational target for secondary mutations is typically much larger than reversion of the original mutation, therefore, secondary mutations are much more likely to occur and be selected,

although reversions can be selected as shown for experimentally evolved *A. baumannii* (Santos-Lopez et al., 2021). Experimental evolution approaches have also been used to decipher possible compensatory changes, that is, evolving already evolved mutants, for example, to explain fitness costs of outer membrane porin loss in different antibiotic resistant mutants (Knopp and Andersson, 2015). However, compensation depends on the environment (Basra et al., 2018), and the trade-offs displayed in a simple setup are not necessarily the same *in vivo* where multiple diverse factors interact with each other (Björkman et al., 2000). If the selective pressure is absent from the experimental setup, the trade-off is not displayed. In this case, experimental evolution can serve to answer why certain mutants are not selected in real life. This is evident from the study by van Ditmarsch et al., where *P. aeruginosa* rapidly evolved hyperswarming in multiple parallel lineages *in vitro*, but lost biofilm formation as a trade-off (van Ditmarsch et al., 2013). They could not detect such hyperswarmers in naturally occurring environmental or clinical isolates, hinting at compensatory evolution to account for the biofilm loss, which is an essential part of the lifestyle of *P. aeruginosa*. In this case, experimental evolution can help identify certain new factors involved in specific phenotypes.

Many infection-related evolution experiments have been performed in planktonic cultures, and they often focus on antibiotic resistance mainly due to its clinical relevance but also because it is a simple way to introduce selection (Björkman et al., 2000; Adler et al., 2014; Knopp and Andersson, 2015; Wistrand-Yuen et al., 2018; Guðmundsdóttir et al., 2021; Santos-Lopez et al., 2021; Eger et al., 2022). Since one can simply change the concentration of the antibiotic added to the growth medium, evolving populations can be compared not only based on the presence or absence of the selective pressure, but also on different strengths of selection. In this case, important findings can emerge, showing that even at low, sub-inhibitory concentrations, high-level antibiotic resistance can be selected (Wistrand-Yuen et al., 2018), hinting that the selection and enrichment of resistant mutants can happen not only at the high concentrations found in the hospitals, but also at low concentrations in the environment.

Simple experimental design can indeed be useful, especially when answering fundamental questions. However, especially in connection with pathoadaptive evolution, one has to acknowledge the limitations of such an approach. Neither our environment nor our bodies resemble constantly shaking flasks with fresh medium supporting growth of homogeneous freely-swimming bacteria. Instead, we are surrounded and defined by diverse surfaces or compartments, different levels of continuous or intermittent flow, and varying nutrient availability. Considering biological relevance, experimental designs have been shifting to more complex environments, although they are still not as common as planktonic setups (Steenackers et al., 2016; Van den Bergh et al., 2018). In an attempt to mimic spatial constraints, Baym et al.

constructed an evolution experiment that is visually pleasing on a macroscale: the huge (120x60 cm) MEGA plate (Microbial Evolution and Growth Arena), which was used to evolve trimethoprim and ciprofloxacin resistance in *E. coli* (Baym et al., 2016). The authors concluded that in contrast to a well-mixed environment, spatial restriction enables maintenance of mutants that are not necessarily the fittest or the most resistant. This illustrates a clear example of clonal interference, as mentioned earlier. In connection with pathoadaptive evolution, spatial constraint is the hallmark of biofilms, as already mentioned earlier and discussed in later sections. Newly arising mutants can be isolated from each other, making the evolving population even more diverse by increasing clonal interference (Gerrish and Lenski, 1998; Steenackers et al., 2016). Parallel experiments in planktonic and biofilm cultures have already started highlighting the environment-dependent selection differences, for example, in antibiotic resistance. *Acinetobacter baumannii* lineages that evolved in a biofilm on beads as opposed to the planktonic phase were more diverse and acquired different mutations conferring lower level resistance to ciprofloxacin, and showed little fitness cost in the absence of antibiotic (Santos-Lopez et al., 2019). Similarly, *P. aeruginosa* that evolved in a colony biofilm model acquired low-level resistance to ciprofloxacin with mutational targets different from its planktonic counterparts (Ahmed et al., 2018).

In addition to studying how different traits would evolve within a biofilm, what about the adaptation to the biofilm lifestyle itself? If biofilms are an inherent part of life inside the host, then mutants with better biofilm capacity could evolve, which is exactly what is observed, for example, in isolates of *P. aeruginosa* or *Achromobacter xylosoxidans* from the CF lungs (Markussen et al., 2014; Khademi et al., 2021). We also show in **Paper II** that genetic variants of the *K. pneumoniae* outbreak clone with enhanced surface-attached biofilm formation arise at infection sites. Therefore, one of the emerging setups for experimental evolution is adaptive evolution during growth in a biofilm (Poltak and Cooper, 2011; Traverse et al., 2013; McElroy et al., 2014; Penterman et al., 2014; Flynn et al., 2016; Steenackers et al., 2016; Mhatre et al., 2020). In **Paper III**, we explored how *K. pneumoniae* would adapt to become a better biofilm former when cycled during growth in a surface-attached biofilm to reflect the common association with the medical devices. Different biofilm models have been used for experimental evolution (Steenackers et al., 2016). The choice of the biofilm growth system introduces more parameters that can potentially affect the outcome of experimental evolution by affecting the bottlenecks, mutation supply, and fluctuation of conditions. For example, in our setup, the biofilms were disrupted every 48 h and exposed to new pegs to impose selection for attachment and biofilm maturation (Figure 3). Most evolved mutants acquired strong biofilm phenotypes without losing the ability to grow well under planktonic conditions, although in other setups loss of growth in liquid was observed for good biofilm formers (Penterman et al., 2014). Another published setup involved growing biofilms on beads, where

the natural dispersal of bacteria from already formed biofilms would inhabit a new bead (Poltak and Cooper, 2011; Traverse et al., 2013; Mhatre et al., 2020). Comparing this setup with ours, one can imagine that maybe the ability to disperse does not play a big role in our setup (**Paper III**) because all populations are dispersed by vortexing every 48 h, while the mutants unable to naturally disperse in a bead model would essentially be a dead-end. Similarly, disruption of the biofilm in our setup can briefly reduce clonal interference during a short time when the disrupted population has to attach again. Collectively, this illustrates a more complex interpretation of the results compared to a simple planktonic culture.

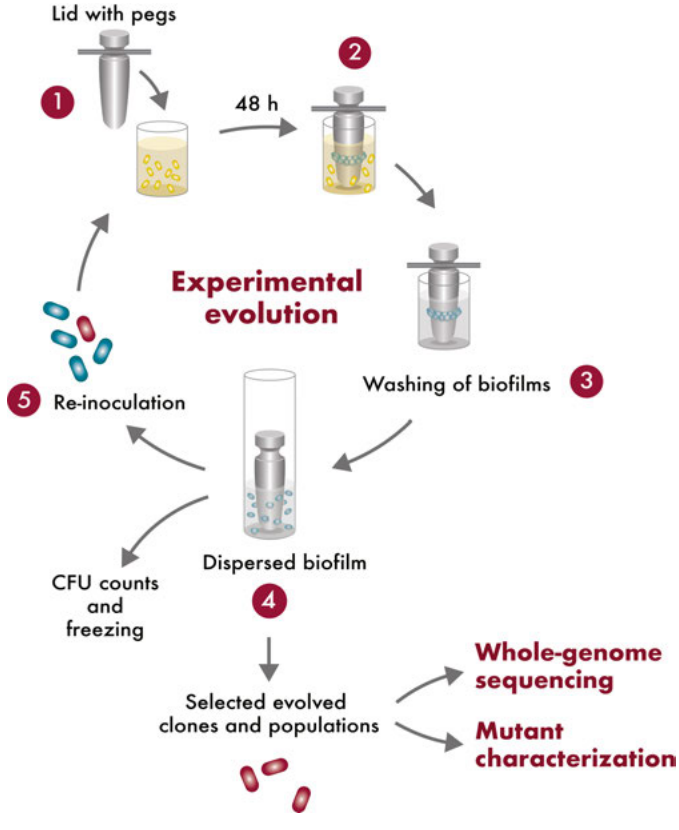

Figure 3 | Experimental evolution setup to select for increased biofilm formation in Paper III. (1) The FlexiPeg lid with pegs was transferred to a 96-well plate with diluted *K. pneumoniae* cultures. (2) Biofilms were allowed to form for 48 h with a change of growth medium after 24 h and (3) they were harvested by vortexing. (4) The dispersed biofilm population was used for CFU counts and (5) re-inoculation of new pegs to start a new cycle.

Biofilm growth alone, like any other lab-based model system, does not replicate the full in-host situation, where multiple host factors interact with the bacteria as well. Therefore, maybe some mutations that would be deleterious

in the host immediately, for example, because of increased susceptibility to immune factors, are maintained *in vitro* because such selection is absent in the lab. Therefore, if the aim is to gain insight into the actual within-host evolution and not just fundamental knowledge of certain processes, it is best to compare it with the real clinical data. For example, we would not have been able to explain why one UTI isolate from the outbreak (**Paper II**) acquired a rare wrinkly morphotype because there were many genetic changes if we had not found the same phenotype and a single overlapping mutation in an experimentally evolved clone (**Paper III**). Other setups to experimentally evolve bacteria have also been described and include certain host factors, for example, serial passaging in macrophages (Miskinyte et al., 2013), more physiological conditions, such as the chronic wound infection model (Vanderwoude et al., 2020), or animal models, for example, evolution during nasal colonization in mice (Cooper et al., 2020).

Although *K. pneumoniae* has been the subject of within-host evolution studies (Jousset et al., 2018; Lee et al., 2019; van Dorp et al., 2019; Wylie et al., 2019; Aihara et al., 2021), experimental evolution studies are scarce. Some studies have looked at the evolution of antibiotic resistance in planktonic setups (Guðmundsdóttir et al., 2021; Zhang et al., 2021; Eger et al., 2022) and the role of nutrient availability for capsule maintenance (Buffet et al., 2021). **Paper III** presents the first experimental evolution of three clinical *K. pneumoniae* strains toward increased biofilm capacity and connects the findings with those from within-host evolution during a large hospital outbreak (**Paper II**) caused by one of the strains studied experimentally. In the following sections, I introduce various structures and processes on which selection can act during different stages of colonization and infection with a focus on specific features of *K. pneumoniae*.

Klebsiella pneumoniae: from colonization to infection

General overview

K. pneumoniae was first introduced to the medical field as a Friedländer's bacillus, named after the German microbiologist Carl Friedländer, who first described it after isolating it from the lungs of patients who died of pneumonia (Friedlaender, 1882). Later, it was renamed after another German bacteriologist, Edwin Klebs, who had observed this bacterium earlier. *K. pneumoniae* is a non-motile Gram-negative bacterium and belongs to the *Enterobacteriaceae* family. With the advance of genetic analysis, *K. pneumoniae* has been divided into multiple distinct phylogroups: first based on *gyrA* sequences (Brisse and Verhoef, 2001) and later based on genomic analysis of >300 *K. pneumoniae* isolates, which supported splitting it into three distinct species (Holt et al., 2015). The most recent consensus is to refer to the *K. pneumoniae* species

complex, which includes closely related species with 95-96% average nucleotide identity, and sharing only 90% nucleotide identity with other *Klebsiella* species (Wyres et al., 2020). The *K. pneumoniae* species complex currently includes *K. pneumoniae*, *K. quasipneumoniae*, *K. variicola, K. quasivariicola,* and *K. africana*. In this thesis, *K. pneumoniae* refers to *K. pneumoniae sensu stricto*.

The high plasticity of the genome is one of the distinguishing features of *K. pneumoniae*. It has been shown to have an open pangenome and a large accessory genome with almost 30,000 protein-coding genes identified so far (Holt et al., 2015). The accessory genes seem to be rare, with one third of them only present in a single genome and likely origin from diverse bacteria, such as other *Enterobacteriaceae*, *Vibrio*, and *Acinetobacter* (Holt et al., 2015). The dynamic nature of the *K. pneumoniae* genome is evident from the numerous plasmids it can carry, for example, averaging 4.5 plasmids per strain in a genomic study of 1,777 ESBL-producing *K. pneumoniae* and showing extremely high diversity (Long et al., 2017). The genes so far reported to be present on such plasmids can include genes for antibiotic resistance, metal resistance, virulence (e.g., the pLVKP plasmid) and biofilm formation (e.g., the *mrk* cluster on pOLA52, c-di-GMP turnover proteins), thus inducing a wide range of phenotypes upon transfer directly affecting survival in the host (Burmølle et al., 2008; Madsen et al., 2012; Ramirez et al., 2014; Gu et al., 2018; Choby et al., 2020). Furthermore, since *Klebsiella* appears to be good at acquiring genes from other sources, the "mix-and-match" type of formation of new gene combinations can arise, as exemplified in the following sections.

Although initially found in pneumonia patients, *K. pneumoniae* is able to cause a wide range of infections, depending on the virulence features of the specific strain/clone and the status of the host. *K. pneumoniae* is typically divided into two pathotypes: classical (cKp) and hypervirulent (hvKp). More widespread cKp strains interact with hosts in an opportunistic manner – they usually colonize the gut first and need facilitating factors to be able to cause infection. Recent whole-genome sequence analysis showed that approximately 50% of *K. pneumoniae* strains isolated from infection sites were identical to those in faeces of the same person (Gorrie et al., 2022). Some examples of factors predisposing to infection by cKp involve underlying diseases, especially compromising the immune status, presence of indwelling medical devices, and treatment with antibiotics (Gorrie et al., 2022). Therefore, not surprisingly, infections caused by cKp are often nosocomial, and can range from localized urinary tract infections, mostly catheter-associated (CAUTI), post-surgical wound infections and ventilator-associated pneumonia (VAP), to systemic life-threatening sepsis (Martin and Bachman, 2018). The majority of infections are UTIs (52.2-66%), followed by pneumonia (15-21.6%) and wound or tissue infections (10-20%) (Long et al., 2017; Gorrie et al., 2022). Consistent with the opportunistic nature, cKp does not seem to be genetically predisposed to cause a certain type of infection, but rather relies on

deterioration of host health status (Gorrie et al., 2022). Even though mostly related to clinical environments, *K. pneumoniae* is also ubiquitous in natural habitats like soil, water, sewage, and the gastrointestinal tract of animals (Bagley, 1985), which has been suggested as a reason why in contrast to other pathogens like *E. coli* or *Shigella*, it does not result in infection-specialized clones. The three strains of *K. pneumoniae* studied in this thesis are all clinical cKp strains, but they were isolated from different sites: IA565 (ST105) from lungs (Hornick et al., 1995), C3091 (ST14) from UTI (Oelschlaeger and Tall, 1997) and the outbreak clone DA14734 (ST16) from a wound, although different variants caused UTIs, blood, and wound infections.

Hypervirulent *K. pneumoniae* (hvKp) refers to pathotypes with a much more invasive nature of infections. In contrast to cKp, they can cause severe infections in otherwise healthy people. The spectrum of infections also differs: pyogenic liver abscesses were the first to be associated with hvKp (Liu et al., 1986) and continue to be dominated by this pathotype, especially in Southeast Asia. Unlike other *Enterobacteriaceae*, hvKp can spread metastatically and cause infections at multiple sites (e.g., brain, eyes, deep tissues) as well as result in bacteremia without an apparent primary site (Russo and Marr, 2019). Although hvKp can cause infection without any predisposing factors, there are indications that it still undergoes a colonization stage, as the same strain was found both in faecal samples and in abscess isolates in the same patient (Fung et al., 2012). However, the differentiation between cKp and hvKp is not straightforward. Quite often, hvKp has been used interchangeably with the hypermucoviscous phenotype and increased capsule production (described further in *Capsule synthesis, transport and regulation*), which is not entirely accurate, as noted in a recent review (Russo and Marr, 2019). While hvKp can indeed have increased capsule production via *rmpA* (regulator of mucoid phenotype) or *magA* (K1-specific *wzy*) leading to devastating catheter clogging or poor drainage in abscesses (Russo and Marr, 2019), it alone does not accurately differentiate the hypervirulent nature of infection. Evaluation of mucoviscosity or the presence of specific capsule types (K1, K2, K5, K20, K54, and K57) does not differentiate strains reliably (Russo et al., 2018). Instead, higher total siderophore production and the presence of *peg-344* (putative transporter), *iroB* (salmochelin siderophore), *iucA* (aerobactin), $_{p}$*rmpA,* or $_{p}$*rmpA2* genes (regulators of the mucoid phenotype on virulence plasmids) have been suggested as more reliable biomarkers to differentiate hvKp from cKp (Russo et al., 2018). However, this approach has been partly questioned for not taking into account population structure and gene structure (e.g., gene linkage) (Wyres et al., 2020). The virulence factors conferring hypervirulence are often encoded on plasmids, such as pLVKP (Choby et al., 2020; Russo et al., 2021). In general, hvKp strains remain rather rare, compared to cKp, and are mostly community-acquired (Gorrie et al., 2022).

Studying infection and colonization experimentally

In vitro assays to study virulence

Certain *in vitro* assays can serve as a proxy for studying infection-related phenotypes mediated by specific factors. One such example is the serum killing assay, which is based on the ability of bacteria to survive complement attacks and thus evade an essential part of the innate immunity. We performed serum survival assays to assess the resistance to complement in outbreak isolates (**Paper II**) and in biofilm-evolved mutants (**Paper III**).

The complement system is a network of soluble and cell-bound proteins, present in tissues and body fluids, in addition to serum (Lambris et al., 2008). There are three major complement activation pathways: classical, lectin, and alternative, and the proteolytic cascade eventually culminates in C3 convertase cleaving C3 into C3b protein that is then deposited on the cell surface (Lambris et al., 2008). This deposition of C3b has multiple unfortunate effects for bacteria: i) phagocytic cells recognize the "labelled" cell surface and initiate phagocytosis, ii) C5 convertase cleaves C5 into C5b initiating the formation of the membrane attack complex (MAC) that leads to lethal pore formation in the outer membrane. Different cell surface structures of *K. pneumoniae* activate different complement pathways: outer membrane porins (like OmpK36) and LPS activate the classical one, and capsule (with mannose or rhamnose residues) in some strains can activate the lectin pathway (Albertí et al., 1993, 1996; Sahly et al., 2009). The alternative pathway serves as an amplification of the classical and lectin ones (Lambris et al., 2008), and the activation of more than one pathway is needed to kill *K. pneumoniae* (Albertí et al., 1993).

The main mechanisms by which *K. pneumoniae* protects itself from the complement system is by physically preventing the complement complex to reach the membrane and by masking the surface components to not activate the complement system (Jensen et al., 2020; Short et al., 2020). While at first it was thought that due to the capsule, complement proteins do not get deposited on the cell surface at all, it has been shown that deposition can occur, but the thickness of the capsule determines the sensitivity or resistance to serum (Jensen et al., 2020). Modification of LPS also affects the susceptibility to complement: smooth LPS due to long O-antigen chains prevent complement protein deposition on the cell surface in contrast to rough LPS lacking O-antigen (Ciurana and Tomas, 1987). However, as Short et al. points out in their study, we are still far from having a full picture of how components studied alone work together, especially since there are strain-dependent (e.g., cKp vs hvKp) differences (Short et al., 2020). The loss of three genes, *rfaH*, *lpp* and *arnD,* was an overlapping explanation for decreased survival in serum; however, 93 genes were affected overall when combining results from all strains (Short et al., 2020). Furthermore, a recent study of 164 clinical isolates of *K. pneumoniae* from three hospitals in Thailand could not find any correlation

between capsule production and serum resistance, and suggested that it is the interplay between different cell surface structures rather than one single factor determining the survival against complement (Loraine et al., 2018).

Survival in macrophages or attachment to epithelial cells can also provide clues about the susceptibility to different components of the immune system and factors needed to establish infection. Attachment to lung and bladder cells was evaluated for some of the evolved mutants with enhanced biofilm phenotype in **Paper III**.

Animal infection models

Infection by *K. pneumoniae* has been extensively studied, often in mice, either to decipher the overall virulence profile or specific infection types, such as UTI or pneumonia (Lau et al., 2007a; Bachman et al., 2011, 2015; Rosen et al., 2018; Russo et al., 2021), and recently reviewed by (Joseph et al., 2021).

One of the approaches applied to study *K. pneumoniae* infection *in vivo* is large-scale screens. Bachman et al. generated a >25,000 transposon mutant library that was used in a murine pneumonia model to identify genes in which loss-of-function causes fitness defects. Of more than 300 mutants, six were further followed and resulted in the conclusions that RfaH, a global transcription antiterminator that regulates LPS and capsule, essential amino acid (branched and aromatic) synthesis, and a copper efflux pump are the most important in this niche (Bachman et al., 2015). Smaller in scale, but also exploratory in nature, was the study by Lau et al., where two *K. pneumoniae* strains, including IA565 studied here, with different virulence phenotypes were studied in a murine pneumonia model (Lau et al., 2007a). Nine genes that differentiated these two strains, hinting at a role in virulence, were connected to iron uptake (siderophore receptors), type 1 fimbriae, the two-component sensor KvgS, later shown to positively regulate capsule production in hvKp (Lin et al., 2006), energy production and conversion, and membrane transport. A slightly different approach was taken by Lai et al. who looked at the induction of various genes during infection, rather than disruption of genes (Lai et al., 2001). However, as Palacios et al. write in their paper: "The repertoire of confirmed *K. pneumoniae* virulence factors has changed little during the past 2 decades" (Palacios et al., 2017), illustrating that few of these large screens are followed up in more detail. Capsule, LSP, fimbriae and siderophores have become a true "mantra" describing the virulence factors in *K. pneumoniae*, but with such intricate regulatory networks, different strain backgrounds, and possible mutational diversity arising during infection and/or colonization, the question becomes not "what" affects the virulence, but "how" exactly. This is also reflected in our studies (**Paper II** and **Paper III**), where mutations selected during the outbreak or experimental evolution can give a completely different phenotype, when present in the same pathway (e.g., capsule) or even the same gene.

More targeted experiments often include competition between “wild-type” and certain mutants or different strains chosen for other phenotypes, for example, biofilm formation. This is especially common in the study of fimbriae in *K. pneumoniae*, and their involvement in UTI or pneumonia (Murphy et al., 2013) or other more detailed analysis of certain genetic constructs, for example, involved in iron acquisition (Palacios et al., 2017).

The assessment of virulence for cKp strains is more challenging than for hvKp, as they do not lead to death in mice and the evaluation relies purely on differences in bacterial count. In addition, it is practically challenging to perform high-throughput experiments in mice. Therefore, alternative models have been implemented to study infection. The greater wax moth *Galleria mellonella* larvae represents a relevant high-throughput method that has been increasingly used to decipher the pathogenicity of many bacteria, including *K. pneumoniae* (Insua et al., 2013; Wand et al., 2013; Bruchmann et al., 2021), and we used the *G. mellonella* larvae model in **Paper II** and **Paper III** for large-scale virulence screenings.

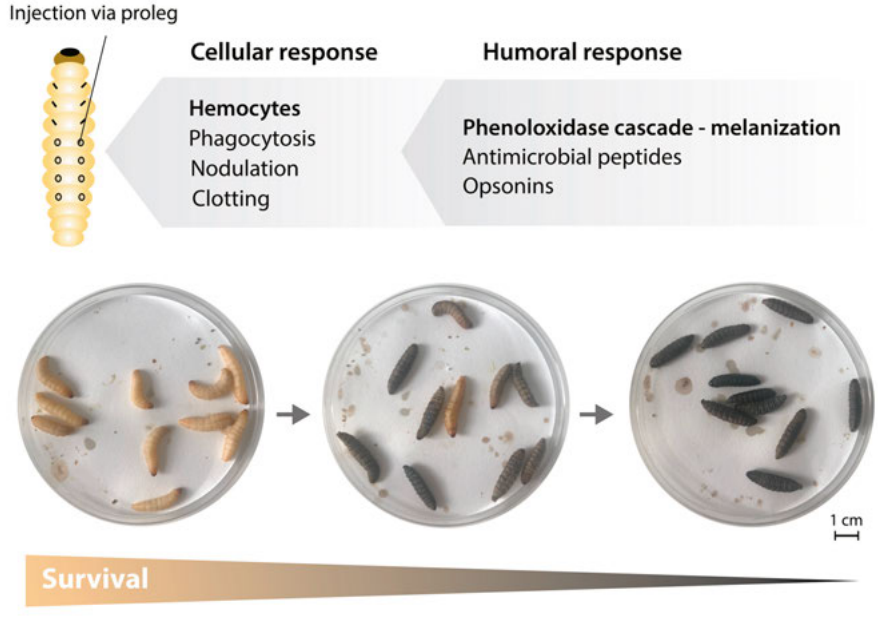

Figure 4 | Overview of the *G. mellonella* wax moth larvae infection model. The immune system has both a cellular and a humoral response that are tightly interconnected. The pictures illustrate the progression of infection after injection of bacterial culture.

Although at first sight the little larvae share little resemblance to humans, their innate immune system, which is the initial barrier all pathogens have to cross, is surprisingly complex and similar to that of vertebrates/mammals (Browne

et al., 2013; Tsai et al., 2016; Sheehan et al., 2018). Both cellular and humoral immunity is present in these larvae (Figure 4). Hemocytes (at least six types) circulate in the hemolymph and adhere to internal organs. Like human neutrophils, certain types of hemocytes phagocytose bacteria, and their numbers increase during infection. In contrast to, for example, nematode models like *Caenorhabditis elegans* that feed on bacteria, these larvae are injected directly via the proleg, and thus the infectious dose is precisely regulated. Moreover, infections can be monitored at 37°C, which is a physiologically relevant temperature to mimic conditions inside the human host. The monitoring itself is assisted by the visual changes reflecting the health status of the larvae: healthy ones are slightly yellow without any discoloration, and due to melanization they become darker and darker as infection proceeds. Melanization is the result of a complex proteolytic cascade that resembles complement system, briefly introduced earlier. The proenzyme is released from the hemocytes upon entry of a pathogen or injury to the cuticle, and proceeds to activate phenoloxidase, which in turn leads to monophenol and phenol conversion to quinines and eventually production of melanin that coats the pathogen (Cerenius and Söderhäll, 2004). The level of darkness can be dependent on the bacterial strain, as I have also seen with strains studied in this thesis, but typically larvae are pitch black when dead. In addition to mortality, health index scores can be given to further differentiate surviving larvae by the extent to which they are affected (Loh et al., 2013). This scoring system is based on a few parameters, such as different levels of activity (movement, response to stimuli) and melanization. Furthermore, immunological parameters to study the larval response to bacteria can also be assessed (Insua et al., 2013; Wand et al., 2013). The use of *G. mellonella* does not require ethical permits and is much cheaper than, for instance, mice. The success of using this infection model depends mainly on how virulent the studied strains are and the quality of the larvae. While we were able to use research-grade larvae for the outbreak screening (**Paper II**), due to the fact that the company closed down after some unfortunate turn of global events (Covid-19 and then Brexit), we had to turn to the regular ones from a reptile feed shop for the evolved biofilm mutants (**Paper III**). As others have observed, the latter usually shows more batch-to-batch variation and therefore requires many more replicates to achieve meaningful results (Champion et al., 2016), especially if the strain is less virulent, like IA565. In **Paper II**, we show that the *G. mellonella* larvae model was powerful enough to differentiate 110 clonal outbreak isolates in terms of different acute virulence properties, sometimes conferred by a single mutation.

Colonization

In contrast to infections, colonization remains a rather unexplored part of the interaction with the host for *K. pneumoniae,* although many studies have been published (Favre-Bonté et al., 1999; Maroncle et al., 2002, 2006; Lau et al., 2008; Perez et al., 2011; Jung et al., 2019; Young et al., 2020). Antibiotic use

in hospitals, in addition to selecting for resistant bacteria, favors colonization with resistant bacteria (Schechner et al., 2011), and antibiotic-driven disruption of the endogenous microflora facilitates colonization with *K. pneumoniae* in mice (Lai et al., 2001; Struve et al., 2003; Perez et al., 2011; Guilhen et al., 2019). The super-shedding phenomenon, where bacteria residing in the gut are excreted in faeces in increased numbers (above 10^8 CFU/g faeces), has been observed in animals that are colonized and/or infected by, for example, *E. coli* O157 (Chase-Topping et al., 2008). This has been suggested to happen due to different localization of bacteria in the large intestine (more towards the terminal part) and directly linked to increased host-to-host transmission (Chase-Topping et al., 2008). The transient super-shedder phenotype can also be promoted by antibiotic treatments, thus increasing host-to-host transmission, as shown for *K. pneumoniae* colonizing the GI in mice (Young et al., 2020). In contrast to approximately 10^4–10^7 CFU/g faeces, the number of bacteria in faeces consistently increased above 10^{10} CFU/g faeces right after streptomycin treatment. Unlike in true enteropathogens, such as *Salmonella enterica* Serovar Typhimurium, this phenotype in *K. pneumoniae* does not induce an acute inflammatory response in the gut (Young et al., 2020). In addition to gut colonization, *K. pneumoniae* can also reside in the nasal mucosa (Podschun and Ullmann, 1998), and Young et al. described that mice orally fed *K. pneumoniae* showed stable GI colonization even without disruption of the gut microbiota (Young et al., 2020). The authors suggested that *K. pneumoniae* might somehow become primed to colonize the GI when in the oral cavity, but the mechanism would need to be further investigated.

Most of the studies to identify the factors needed for colonization involved negative screening after generating transposon mutant libraries (Maroncle et al., 2002). While it can help identifying the important factors, such approaches are biased towards loss-of-function changes, and do not necessarily reflect the genetic diversity arising during actual colonization. The bacterial factors identified as important for successful colonization of *K. pneumoniae* vary depending on the strain and experimental setup. For example, signature-tagged mutants identified as attenuated in colonization in streptomycin-treated mice (Struve et al., 2003) were completely different from those found in ampicillin-treated mice (Lai et al., 2001), which might reflect the importance of competition with different microflora. The capsule seems to be essential in *K. pneumoniae* colonization of both the oral cavity and the gut in mice that had not been treated with antibiotics (Young et al., 2020), while the role of capsule is less clear during colonization of the GI with antibiotic-disrupted microflora. A recent study showed that capsule can be costly to produce during gut colonization and that a hvKp capsule null mutant even outcompeted the wild-type in ampicillin-treated mice (Tan et al., 2020). Other factors identified as important are various metabolic genes, such as urease, glutamate synthase, type 3 fimbriae, membrane transporters, and other genes, although very few are studied in more detail (Maroncle et al., 2006; Joseph et al., 2021). In some

cases, the screens were accompanied by *in vitro* adhesion assays with intestinal epithelial cells; however, the results did not really correlate with the colonization potential (Maroncle et al., 2002). This reflects that diverse sets of functions are needed for *K. pneumoniae* to establish itself at a colonization site.

In **Paper II**, we explore how genetic variants of the same *K. pneumoniae* clone with various acquired mutations and phenotypes colonize the GI of mice with and without antibiotic treatment (clindamycin). We identified that increased biofilm phenotype enhanced colonization, while the role of capsule was dependent on the exact mutation (YrfF vs Wzc and ManB).

Capsule synthesis, transport and regulation

The "sugary" coating that provides *K. pneumoniae* colonies with their distinct mucoid/slimy appearance is one of the most, if not the most, studied features of *K. pneumoniae*. It is not surprising, considering the essential role of capsule in multiple infection-related phenotypes. Changes from mucoid (with capsule) to non-mucoid (lost capsule) phenotypes are common in clinical isolates of *K. pneumoniae* (Lee et al., 2019; Chiarelli et al., 2020; Ernst et al., 2020), possibly reflecting adaptations to a certain niche or stage of infection. We discuss numerous genetic changes in capsule both during a hospital outbreak in **Paper II**, and during the evolution of increased biofilm formation in **Paper III**.

Capsular polysaccharides (CPS), also called K antigens, are acidic polysaccharides of high molecular weight. *K. pneumoniae* has a wide variety of capsule types that vary in the sugar content and linkages between them, determined by the activity of glycosyltransferases. More than 70 capsular types of *K. pneumoniae* have been determined by serotyping, and recent genetic analysis of different *cps* clusters (K loci) revealed the presence of >130 different capsular locus (KL) types (Wyres et al., 2016). *K. pneumoniae* isolates from infection sites can have a wide range of capsule types even in the same hospital (Long et al., 2017). Based on the fact that *K. pneumoniae* is also ubiquitous in the environment (Bagley, 1985), it was suggested that CPS might be under a strong diversifying selection not only because of the exposure to host factors, but also as protection from phages and protists (Wyres et al., 2016). Similarly, a recent study by Buffet et al. suggests the maintenance of capsule as an environmental adaptation, since the capsule was not lost as frequently in nutrient-poor environment during serial passaging (Buffet et al., 2021). The three *K. pneumoniae* strains included in this thesis belong to different KL types as determined by the Kaptive database (Wyres et al., 2016; Wick et al., 2018). The polysaccharide structures for two of them are known: K16 (as for the C3091 UTI isolate) is composed of repeating tetrasaccharides of D-galactose, D-glucose, L-fucose and D-glucuronic acid residues (Chakraborty et al., 1977), while the tetrasaccharide repeat unit of K51 (like for the DA14734 outbreak

isolate) is composed of D-glucuronic acid, D-glucose, and two D-galactose residues (Chakraborty et al., 1982). The respiratory isolate IA565 is assigned to capsule type KL102 (previously KN2 type), but the polysaccharide structure has not yet been determined.

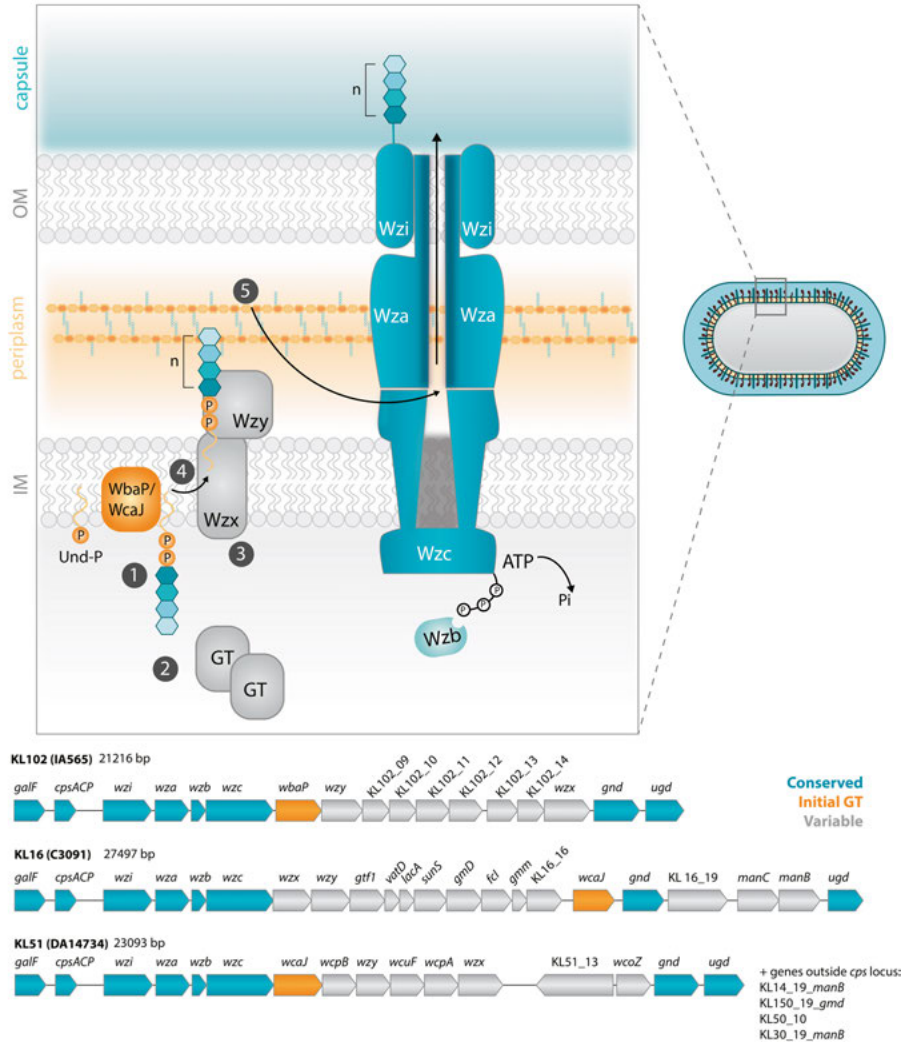

Figure 5 | Schematic representation of capsule synthesis and export loci and machinery in *K. pneumoniae.* (1) Glucose or galactose is added to undecaprenyl phosphate by the initial glycosyltransferase WcaJ or WbaP. (2) Additional capsule type-dependent glycosyltransferases (GT) build the capsule repeat unit by inserting different monosaccharides. (3) Wzx flippase moves the lipid-linked precursor across the inner membrane to the periplasmic side. (4) The capsule chain is then elongated by Wzy polymerase and transported by Wzc tyrosine autokinase (cyclically dephosphorylated by Wzb phosphatase) and Wza, then it is attached outside, at least initially, to Wzi.

The genes responsible for capsule synthesis and export are encoded in a *cps* locus that can vary in size between 10-30 kbp (Wyres et al., 2016). The *cps* loci for the three *K. pneumoniae* strains included in this thesis are shown in Figure 5 (bottom). The 5' end of *cps* with six initial genes is usually conserved as is the 3' end with *gnd* and *ugd*, but the central region between *wzc* and *gnd* is highly variable (Shu et al., 2009; Pan et al., 2015). Due to their conserved function but genetic polymorphism, the nucleotide sequences of *wzc* and *wzi* can be used to differentiate different KL types (Brisse et al., 2013; Pan et al., 2013), but the Kaptive database, taking into account the entire *cps* locus organization and sequence homology for each gene, seems to be superior (Wyres et al., 2016; Wick et al., 2018). LPS synthesis genes can overlap with those involved in capsule synthesis, as they require the same sugar precursors. For instance, mannose is part of the O3b antigen structure in DA14734, and fucose, for which mannose is an intermediate synthesis step, is part of CPS in this capsule type. In fact, *manB* and *manC* genes, responsible for these mannose synthesis steps, are found outside the *cps* cluster in this strain, but inside the main one in C3091. In the case of DA14734, the genes outside the *cps* cluster actually have a high sequence identity to the corresponding genes from completely different KL types, according to the Kaptive database. This illustrates how *cps* loci are highly prone to genetic variation via recombination. Point mutations, which we focus on in **Paper III** especially, and to some extent IS element insertions, are also frequent, for example, in Wzc, Wzb or the initial glycosyltransferase WbaP.

Capsule synthesis is orchestrated by a giant macromolecular machinery, and in *K. pneumoniae* it is carried out via the Wzy-dependent pathway (Figure 5), like *E. coli* group 1 capsules and some exopolysaccharides in other bacteria (Whitfield, 2006; Whitney and Howell, 2013; Whitfield et al., 2020). Synthesis is initiated on the cytoplasmic side of the inner membrane, where the initial glycosyltransferase (WbaP or WcaJ) attaches the first glycosyl residue to undecaprenyl phosphate (Und-P). Then, depending on the capsular type, diverse soluble or membrane-bound glycosyltransferases complete the synthesis of a repeat unit. The linked building block is then flipped across the membrane by the Wzx flippase protein, presenting it to the Wzy polymerase to grow the polymer chain. The regulation of chain length and export is coupled together and is mediated by the Wzc tyrosine autokinase. Wzc is an inner membrane-spanning co-polymerase that undergoes cyclical autophosphorylation on the C-terminal tyrosine-rich tail, which is dephosphorylated by the cytoplasmic phosphatase Wzb (Preneta et al., 2002). The periplasmic part of Wzc also interacts with the Wza transporter to move the assembled polymer outside the cell (Collins et al., 2007). Unlike excreted polysaccharides (e.g., colanic acid in *E. coli*), capsule polysaccharides are typically attached to the outer cell surface. It is suggested to occur through the Wzi lectin, at least in the initial stages of capsule assembly (Bushell et al., 2013), as the knock-out of *wzi* leads to capsule secretion rather than retention on the cell surface (Rahn et al., 2003).

A detailed structural analysis recently showed that dephosphorylated Wzc exists as an octamer and dissociates into monomers upon phosphorylation, and that cycling between the two forms is needed for this key step in capsule synthesis and export (Yang et al., 2021) as suggested earlier (Wugeditsch et al., 2001). This presents an essential step in shaping the final polymer chain, but also in making sure that it actually reaches its final destination on the outer part of the outer membrane. Thus, not surprisingly, the regulation by Wzc has been noted as one of the major unanswered questions in capsule synthesis (Whitfield et al., 2020). Wzc is one of the key proteins in this thesis in **Paper II** and especially in **Paper III**, where we explore the effects of rapidly arising single point mutations, conferring an atypical hypermucoid phenotype that increases susceptibility to human serum, but confers a strong advantage in biofilm, depending on the strain. No connection between hypermucoviscosity and point mutations in Wzc in *K. pneumoniae* was reported before the work presented in this thesis. However, due to the conserved function of Wzc in many bacterial species, there have been reports on the production of high molecular weight polysaccharides in *A. baumannii* (Geisinger and Isberg, 2015). In the *A. venetianus* RAG1 strain, different point mutations in *wzc* led to production of higher molecular weight emulsans, which are used commercially, and their properties (e.g., hydrophobicity) differed from the wild-type polymer (Dams-Kozlowska and Kaplan, 2007). In parallel with our work, Ernst et al. recently described how widespread Wzc mutations are in clinical ST258 isolates conferring a hypermucoviscous phenotype (Ernst et al., 2020), illustrating that genetic variation in *wzc* is important in other strains, too.

The initial stages of capsule synthesis overlap with those of other surface glycan synthesis. Therefore, the study of capsule by gene inactivation is sensitive to the specific gene deleted not because of polar effects on expression, but due to sequestration of Und-P, onto which the entire CPS polymer chain is assembled, or to the toxic build-up of partly synthesized "dead-end" intermediates (Jorgenson and Young, 2016; Tan et al., 2020). Und-P is a common initial lipid carrier molecule in the synthesis of multiple cell-envelope structures, including peptidoglycan, O antigen (LPS), and enterobacterial common antigen, whose synthesis starts on the cytoplasmic side of the inner membrane while linked to Und-PP, and then flipped across, releasing Und-P for a new synthesis cycle. Therefore, in addition to losing capsule, deletion of genes involved in the later stages of capsule production (e.g., *wza* or *wzy*), the whole cell envelope and cell shape becomes distorted (Tan et al., 2020). Deleting the initial glycosyltransferase (WcaJ or WbaP, depending on capsule type) alleviates these defects. The same authors have shown that it is not the capsule loss *per se*, but the distortions in the cell envelope that made the mutants sensitive to bile salts, since the *wcaJ* KO survived as well as the WT, but not the *wza* or *wzy* KO (Tan et al., 2020).

Consistent with the importance of capsule, its synthesis is highly regulated at multiple levels and under diverse conditions (Walker and Miller, 2020).

Multiple two-component systems affect capsule synthesis, such as BarA-UvrY, KvgAS or Rcs (Lin et al., 2006; Dorman et al., 2018; Wall et al., 2018). A key role is played by the Rcs (regulator of capsule synthesis) system, which is a complex machinery involved in the envelope stress response sensing LPS or peptidoglycan defects (Wall et al., 2018; Mitchell and Silhavy, 2019). The sensory lipoprotein RcsF transfers the signal to the sensory histidine kinase RcsC, inducing the phosphorelay cascade to the phosphotransferase RcsD and then to the response regulator RcsB. RcsB then can form heterodimers with other proteins, for example, with RcsA, to induce the expression of capsule as well as some biofilm-related genes and downregulate motility (Flores-Kim and Darwin, 2014). In *K. pneumoniae*, RcsB-RcsA bind upstream of the *galF* promoter, the first gene in the *cps* cluster (Peng et al., 2018). The point mutation in the periplasmic part of the RcsD phosphotransferase was one of the capsule-affecting mutations selected for an increased biofilm phenotype in **Paper III**. Furthermore, RcsB can form homodimers that upregulate the RprA sRNA to induce the stationary phase sigma factor RpoS, mediating protection against various stresses (Mitchell and Silhavy, 2019). In **Paper II**, we also observed an increased capsule mutant with a 21 nt in-frame deletion in *yrfF*/*igaA*, which negatively regulates the Rcs system in, for instance, *Salmonella* (Costa et al., 2003; Tierrez and García-Del Portillo, 2004). Deletion mutations in *yrfF* were also found during experimental evolution in macrophages (Miskinyte et al., 2013) and isolated from keratitis (Van Tyne et al., 2016), suggesting the pathoadaptive nature of such changes.

The nutritional availability also affects the capsule status. A recent study explored the need for capsule depending on the nutrient status in the environment, and showed that *K. pneumoniae* lost capsule more frequently in nutrient-rich conditions *in vitro* (Buffet et al., 2021). Part of the explanation could be iron-dependent regulation. Capsule production is also regulated by global regulators in connection with iron availability, where capsule synthesis is decreased in iron repleted conditions due to increased Fur activity (Lin et al., 2011). In addition, IscR, a Fe-S cluster assembly regulator, also regulates capsule synthesis (Wu et al., 2014). Deletion of H-NS results in derepression of *galF*, *wzi*, *manC,* and the capsule regulator *rcsA* (Ares et al., 2016). Some studies have attempted to identify the global network of regulators more systematically. For instance, Dorman et al. generated transposon insertion mutants in two *K. pneumoniae* strains (K1 and K2 capsule type) and differentiated them as low-capsule or high-capsule mutants after Percoll density gradient centrifugation (Dorman et al., 2018). The results reflected the complex network of genes that can have an impact on capsule production, ranging from genes of *cps* locus itself, LPS genes, two-component systems (BarA-UvrY, RcsAB) to global regulators (H-NS) and metabolic genes. It would be more accurate to say that this study measured the impact on mucoviscosity, as discussed below.

The hypermucoid phenotype in *K. pneumoniae* has been historically associated with virulence plasmids, such as pLVPK or pKP100, encoding the RmpA and RmpA2 proteins that activate capsule production via RcsB and are directly linked to invasive disease by hvKp (Nassif et al., 1989; Cheng et al., 2010). While capsule production (measured by uronic acid quantification) in many cases directly correlates with increased mucoviscosity (i.e., poor sedimentation or positive string test (Fang et al., 2004)), it is possible to have increased mucoviscosity without increase in capsule, as shown in recent studies (Walker et al., 2019; Mikei et al., 2021). Walker et al. showed that the loss of the novel regulatory protein RmpC decreased capsule production but not the hypermucoid phenotype (Walker et al., 2019). In the recent study by Mikei et al., hypermucoviscosity was suggested to require the function of metabolic genes, encoding proteins for the TCA cycle, pyruvate metabolism and energy acquisition, in addition to capsule production itself, reflecting an interconnection between metabolism and cell surface features. However, the mechanisms by which exactly this is achieved, remain to be investigated. The downside of the above-mentioned studies (Dorman et al., 2018; Mikei et al., 2021) is again the analysis of deletion mutants only, when we know from our data in **Paper II**, **III** and **IV** that the phenotype of a deletion mutant can be completely different from that conferred by point mutations in the same gene.

Iron acquisition and metabolism

> "Life is wedded to iron – for better or for worse." Andrews et al., *Metallomics and the Cell*, 2013.

With a few exceptions, iron is essential for the survival and growth of many bacterial pathogens in the human host. Iron is required by different proteins, such as cytochromes, catalases, and peroxidases, and iron-dependent regulation of numerous genes is common. High amounts of free iron are toxic under aerobic conditions due to hydroxyl radical formation; therefore, most of the time it is found bound to other molecules. This is also one of the strategies by the human host to ensure that iron is not easily accessible to bacteria, thus providing "nutritional immunity" (Weinberg, 1975). Some conditions, such as sickle-cell anemia or malaria, result in destruction of heme and the release of iron, and they have been shown to predispose to bacterial infections (Payne and Neilands, 1988). Similarly, people who are treated for iron deficiencies or do not have the right levels of transferrin (thus unable to bind excess iron) have a higher risk of bacterial infections simply because there is plenty of iron to support bacterial growth. Most of the iron is bound to other molecules inside the cell (e.g., ferrous iron Fe^{2+} is part of heme or Fe^{3+}bound to ferritin) or outside (e.g., ferric iron Fe^{3+} is bound to transferrin in serum and lactoferrin in saliva, milk, and tears), and typically the concentration of free iron in the

blood is magnitudes lower than what bacteria require for growth (Payne and Neilands, 1988). So how do they manage to overcome this problem?

One of the major ways is to "steal" the bound iron from host molecules by secreting iron-chelating molecules – siderophores – that are able to bind iron with higher affinity than host proteins, and then bring the iron inside the bacterial cell (Figure 6). Siderophores represent key virulence factors in *K. pneumoniae*, as already mentioned in the section on hvKp (Russo et al., 2021). *K. pneumoniae* can secrete multiple types of siderophores, depending on the pathotype: cKp typically only has catecholate-type enterobactin (Ent), which has the highest affinity for iron and is transported into the cell by the FepABC/Fiu/IroN/Cir systems. However, Ent is inhibited by the innate immunity protein lipocalin 2 (Lcn 2) (Goetz et al., 2002), which is upregulated during, for example, lung inflammation (Cowland et al., 2003) and is part of defense mechanisms against *K. pneumoniae*-mediated lung infections (Chan et al., 2009; Bachman et al., 2011). Salmochelin is a C-glucosylated version of enterobactin, which is no longer inhibited by Lcn2 (Hantke et al., 2003; Fischbach et al., 2006). The ability to produce multiple additional siderophores (aerobactin, colibactin, salmochelin and yersiniabactin) is one of the key features of hvKp and directly links the ability to cause invasive infections (Holt et al., 2015; Russo and Marr, 2019). Yersiniabactin was shown to be the hallmark of *K. pneumoniae* strains that can cause severe respiratory infections due to evasion of Lcn 2 and interestingly, siderophores were not needed for growth in urine in the laboratory (Bachman et al., 2011). Typically, bacteria themselves produce siderophores non-ribosomally and secrete them (e.g., enterobactin via EntS and TolC), but they can also take advantage of other ones circulating around. For example, *K. pneumoniae* (like the strains studied here) can have an uptake system for the hydroxamate-type siderophore ferrichrome (FhuABCD), but the siderophore itself is produced by fungi. Ferrichrome is not inhibited by Lcn 2, thus providing an advantage in strains that produce enterobactin and illustrating the expanded arsenal for such an essential process. In addition, siderophore-independent iron uptake systems exist, although they are used to a much lower extent. For example, the FeoABC system in the inner membrane allows Fe^{2+} to be transported after it passes through the general outer membrane porins (e.g., OmpK36) (Page, 2019). Additionally, some bacteria, like *K. pneumoniae*, can have hemolysins that, as the name implies, lead to heme lysis releasing iron or heme that can be taken up (Kronstad and Caza, 2013). Diversification in iron acquisition is common during within-host evolution. A study in *P. aeruginosa* showed that during long-term adaptation to CF lungs, bacteria evolved towards increased heme utilization from hemoglobin, as opposed to other iron scavenging strategies (Marvig et al., 2014). Various ways to obtain iron were also reported in a globally disseminated *K. pneumoniae* ST258 with increased expression of hemin transport in an *entS* mutant (Holden et al., 2018).

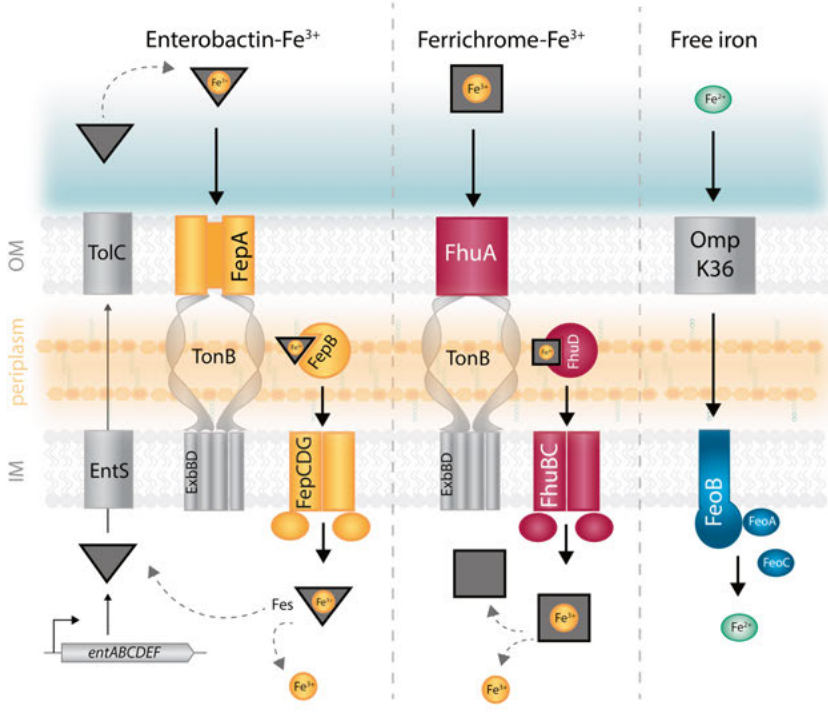

Figure 6 | *K. pneumoniae* iron uptake systems for iron bound to siderophores, such as catecholate-type enterobactin (self-produced) or hydroxamate-type ferrichrome (produced by fungi), and free iron.

Iron is often incorporated into proteins in the form of Fe-S clusters. In enterobacteria, two main systems coordinate this process depending on the conditions: ISC (*iscRSUA-hcsBA-fdx-iscX*) as a normal housekeeping system, and SUF system (*sufABCDSE*) that functions more during oxidative stress and iron starvation (Ayala-Castro et al., 2008). Both systems are regulated by the global regulator Fur (ferric uptake regulator) and small RNA RyhB. When there is plenty of iron, it is bound by Fur and leads to repression of genes that are needed for iron uptake. In contrast, when iron is scarce, repression is alleviated. In addition, RyhB ensures that during iron-depleted conditions, iron-containing proteins are not produced as much to save it for the most important processes. In addition, IscR, a Fe-S cluster protein itself acts as a global regulator as well leading to activation of transcripts that are repressed by Fur (Ayala-Castro et al., 2008). Involvement of iron status in regulatory pathways is essential for many processes, including those mediating virulence and biofilm formation. There are indications that some regulation might be specific to UPEC, for example, increased amino acid uptake as opposed to synthesis in the iron limiting-environment of the urinary tract (Banerjee et al., 2020). In *K. pneumoniae*, Fur has been shown to indirectly repress capsule via *rcsA*

during iron-replete conditions (Lin et al., 2011) and regulate type 3 fimbrial expression via *mrkHI* (Wu et al., 2012). IscR is also a global regulator that autoregulates its own expression along with the whole *isc* system, described above. Its connection to capsule (Wu et al., 2014), type 3 fimbriae (Shen et al., 2017), and type 1 fimbriae (Wu and Outten, 2009) has also been shown. However, both Fur and IscR regulons are massive, based on detailed analysis mostly in *E. coli* (Andrews et al., 2013; Seo et al., 2014; Banerjee et al., 2020), and many more functions are likely to be assigned in the future. Bacterial interactions in polymicrobial biofilms can also be dependent on iron status, for example, *E. faecalis* inhibits *P. aeruginosa* when iron is scarce by producing lactic acid and reducing pH (Tan et al., 2022).

We find that multiple iron uptake genes (siderophore-dependent: *fhuA, fepA, fiu* or independent: *feo*) or Fe-S synthesis genes (*sufABCDS*) were under positive selection during the hospital outbreak in **Paper II**. Some of those changes (e.g., *fhuA*) were present exclusively in isolates with attenuated virulence in *G. mellonella* larvae, and a specific *sufB* mutation was likely to be connected to increased virulence due to better survival under low iron conditions. Other studies have also found iron metabolism (*sufB* mutations) as a trait in persistent *K. pneumoniae* ST258 clones (Marsh et al., 2019). During a recent screen for virulence defects, a *fepB* (periplasmic chaperone for enterobactin/salmochelin uptake) deletion mutant, and more detailed genetic constructs revealed that this import protein was more critical for infection than the siderophore synthesis itself (Palacios et al., 2017). Overall, these findings reflect the essential role of iron in life inside the host, and the fact that a lot still remains unknown.

Antibiotic resistance

One of the strongest challenges that bacteria encounter in the host, even if they manage to overcome the host defense barriers, is antibiotic treatment. Development of antibiotic resistance is the clearest example of pathoadaptive evolution in action (Davies and Davies, 2010). There are even examples of antibiotic resistance selected as a result of treatment with non-antibiotic compound, e.g., cancer drugs (Guðmundsdóttir et al., 2021).

Antibiotic resistance can arise due to mutations in chromosomal genes and can be acquired horizontally, for instance, on plasmids, where resistance genes can also be mutated further. Possible resistance mechanisms range from general ones like preventing access to the antibiotic target (e.g., porin loss or upregulation of efflux pumps) to more specific i) mutational target alterations (e.g., penicillin binding proteins for beta-lactams, ribosome changes in tetracycline or macrolide resistance, LPS in colistin resistance), ii) modifications of the target (e.g., methyltransferases in aminoglycoside or macrolide resistance), and iii) antibiotic degrading enzymes (e.g., beta-lactamases) (Blair et al., 2015). In terms of mutational resistance, the spectrum of mutations

selected can depend on the antibiotic itself, concentration of antibiotics, and thus the strength of selection (Andersson and Hughes, 2014; Wistrand-Yuen et al., 2018) or the bacterial growth state, for example, biofilm vs planktonic (Zaborskyte et al., 2017; Ahmed et al., 2018; Santos-Lopez et al., 2019). As discussed earlier, resistance often comes with a cost; however, it is highly dependent on the actual mechanism (Andersson and Hughes, 2010). Structural variations, such as gene amplifications, represent a typically unstable mechanism to increase resistance, as they are frequently lost in the absence of antibiotic pressure (Sandegren and Andersson, 2009), and they are a common genetic basis for heteroresistance (Nicoloff et al., 2019). Some more general resistance mechanisms, for example, porin loss, can have pleiotropic effects and complex trade-offs (Knopp and Andersson, 2015; Phan and Ferenci, 2017). In addition, the cost of resistance can be dependent on the environment. For example, the frequency of mecillinam resistance mutations is extremely high in laboratory experiments, but only *cysB* mutants are found in clinical isolates (Thulin et al., 2015). Resistance plasmids are typically less costly than alterations of essential bacterial processes by mutations (Vogwill and Maclean, 2015). Specific resistance genes can impose a significant cost to the cell (Rajer and Sandegren, 2022), and they can be eliminated by deletions.

Being mainly a nosocomial pathogen, *K. pneumoniae* is frequently exposed to antibiotics both during colonization and different infections. The hospital environment acts as a true "breeding ground" for the development and transmission of resistant *K. pneumoniae* (David et al., 2019). Beta-lactams represent the largest and most widely used group of antibiotics and the ability to produce different types of beta-lactamases is one of the distinguishing features of the globally disseminated *K. pneumoniae* STs, such as carbapenem-resistant ST258 (Bush, 2010; Schultsz and Geerlings, 2012; Jacobs et al., 2014). The presence of extended-spectrum beta-lactamases (ESBL) has been directly linked to increased nosocomial transmission of *K. pneumoniae* (Gorrie et al., 2022). Resistances to all clinically used antibiotics against *K. pneumoniae* have been reported (Navon-Venezia et al., 2017), placing resistant *K. pneumoniae* infections (third-generation cephalosporin and carbapenem-resistant) among those hardest to treat both in Europe (Cassini et al., 2019) and worldwide (Murray et al., 2022). The incidence of resistant infections has been increasing at an alarming rate. For example, the number of infections caused by third-generation cephalosporin and carbapenem resistant *K. pneumoniae* in Europe doubled during 2007-2015 (Cassini et al., 2019). *K. pneumoniae* has been included among the six highest priority pathogens, together named as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) to illustrate how they frequently "escape" the effects of antibiotics due to high antibiotic resistance rates (Rice, 2008). The World Health Organization has classified *K. pneumoniae* as a "critical priority" pathogen in urgent need of new treatment options (World Health Organization, 2017).

Antibiotic resistance in *K. pneumoniae* can be mediated both by chromosomal mutations and the acquisition of resistance genes on plasmids; however, the latter is much more common today (Ramirez et al., 2014; Navon-Venezia et al., 2017). For example, a recent large-scale genomic study of *K. pneumoniae* isolates determined that up to 67.5% of antibiotic resistance genes were plasmid-borne in contrast to 8% of chromosomal genes (the remaining fraction could not be accurately assigned) (Gorrie et al., 2022). Biofilms have also been suggested to contribute to maintenance of plasmids due to fewer cell divisions and consequently a lower loss rate of the plasmids (Madsen et al., 2012). In most cases, the plasmids carry multiple resistance genes, thus creating a bimodal distribution of resistant subpopulations among *K. pneumoniae* strains: either they carry many (5-10) resistance genes, or none (Wyres et al., 2020). Quite a few new antibiotic resistance mechanisms were first described in *K. pneumoniae* before being found in other species (Navon-Venezia et al., 2017). While community-associated or environmental isolates of *K. pneumoniae* can be equally pathogenic as those found in hospitals (Struve and Krogfelt, 2004), nosocomial isolates are linked to carriage of a higher number of antibiotic resistance genes (Holt et al., 2015). Typically, the multidrug resistance (MDR) phenotype is associated with cKp, most likely due to high exposure to antibiotics in the hospital environment. However, considering that both MDR (resistance to >= 3 antibiotic classes in addition to ampicillin) and the hv phenotype are mediated by plasmids, it was only a matter of time before they converged producing a terrifying combination of an extremely virulent *K. pneumoniae* resistant to most available antibiotics (Chen and Kreiswirth, 2018; Gu et al., 2018). For example, a clonal hospital outbreak of a ST11 carbapenem resistant strain (bla_{KPC-2}) with the acquired pLVPK-like virulence plasmid in China caused the death of five patients due to severe pneumonia or sepsis (Gu et al., 2018). Even though the strains appeared to be sensitive to tigecycline and polymyxin B *in vitro*, prolonged treatment with these antibiotics did not eradicate bacteria from the blood. In addition, hybrid plasmids combining both resistance and virulence genes on the same plasmid have also been reported (Shankar et al., 2022). MDR clones seem to be more prone to horizontal gene transfer than hypervirulent ones (Wyres et al., 2019b); therefore, the strategy to stop these hybrids probably should be aimed at prevention of MDR *K. pneumoniae*.

The pUUH239.2 plasmid (Figure 7), present in the *K. pneumoniae* clone that caused the first ESBL outbreak in Scandinavia (Lytsy et al., 2008) is an example of the highly dynamic resistome of *K. pneumoniae* (Sandegren et al., 2012). pUUH239.2 is the result of recombination between two plasmids: the main backbone originates from the plasmid pKPN3 from a *K. pneumoniae* ST258 and the resistance cassette shows a high similarity to the resistance cassette from the plasmid pEK499 from an *E. coli* ST131. Both *K. pneumoniae* ST258 (Pitout et al., 2015) and *E. coli* ST131 (Nicolas-Chanoine et al., 2014) represent globally disseminated high-risk clones. pUUH239.2

carries a huge resistance cassette of approximately 41 kb with genes conferring resistance to macrolides [*mphR*(A), *mrx* and *mph*(A)], trimethoprim (*dhfrXII*), β-lactams (bla_{TEM-1}, bla_{OXA-1}) including extended spectrum β-lactams ($bla_{CTX-M-15}$), sulphonamides (*sul1*), aminoglycosides (*aadA2*), aminoglycosides/fluoroquinolones (low level, *aac(6')-1b-cr*) and tetracycline (*tet*(A) and *tetR*). The presence of IS26 elements makes the resistance cassette prone to various structural rearrangements, for example, amplifications or deletions. In **Paper II**, we explore the evolution of the multidrug resistant (MDR), ESBL-producing *K. pneumoniae* clone encoding pUUH239.2 during a hospital outbreak, where one aim is the analysis of further antibiotic resistance selection and analysis of antibiotic treatment pressures.

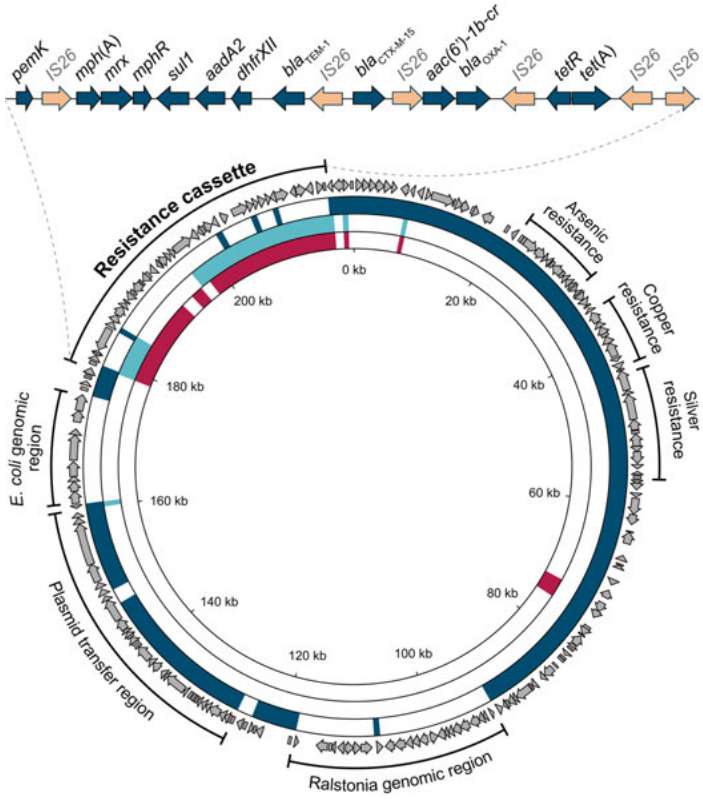

Figure 7 | Multidrug-resistance plasmid pUUH239.2 and its resistance cassette. The main backbone of the plasmid originates from pKPN3 (dark blue), while the resistance cassette shows similarity to the *E. coli* plasmids pEK499 (light blue) and pC151a (burgundy).

Biofilms as a predominant form of bacterial growth

> "The number of these animicules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom." Anthony van Leewenhoek describing microbial growth in dental plaque biofilm in 1684.

Why should we care about biofilms?

Bacterial biofilms broadly describe basically anything that is not freely swimming (planktonic): bacteria that are attached to each other or to a biotic or abiotic surface and enclosed in an extracellular matrix (ECM). This presents an overwhelming variety of lifestyles if one considers i) association to surface (surface-attached vs non-surface attached aggregates or pellicles, ii) the type of surface (biotic – tissues or abiotic – any kind of surface that is not alive), iii) the possible environmental conditions, and iv) all the species or strain-dependent differences. In **Paper III**, we also show how different mutations specifically contribute to biofilm formation in experimentally evolved *K. pneumoniae*.

Although this thesis is focused on biofilms with connection to infections, biofilm formation is universal and is not confined to pathogenic bacteria (Hall-Stoodley et al., 2004). In fact, more systematic studies recognizing bacterial growth as biofilms first appeared in environmental biology. The term "biofilm" was mentioned in a publication for the first time in 1975 to describe a mixed growth of bacteria, protozoa and algae on wastewater filters (Mack et al., 1975). The recognition of biofilm importance in the medical field was initiated by Niels Høiby, when describing the aggregates of *P. aeruginosa* in the lungs of cystic fibrosis patients (Høiby and Axelsen, 1973), and the term "biofilm" was introduced into medical microbiology by John William Costerton in a publication on *P. aeruginosa* biofilm formation on urinary catheter material and its ability to survive extremely high concentrations of the antibiotic tobramycin (Nickel et al., 1985).

General biofilm features

The assembly and growth of bacteria in a biofilm include many different steps requiring different factors orchestrating the attachment, growth, production of ECM, and dispersal. While it is typically presented as a simple linear sequence of events, mostly based on strictly controlled biofilm experiments *in vitro*, it is complex, with multiple stages possible in the same infection or colonization site (Sauer et al., 2022). Adding to the complexity is the difference between different species or even strains and dependence on environmental conditions, such as nutrient status, pH, oxygen availability, and others. Genetic variation, as we show in **Papers II**, **III** and **IV**, can influence the biofilm phenotype in drastic ways as well. In this section, I introduce some of the common features

of biofilms, and then go onto more detailed descriptions of the processes and structures that are the most relevant for this thesis and for *K. pneumoniae*.

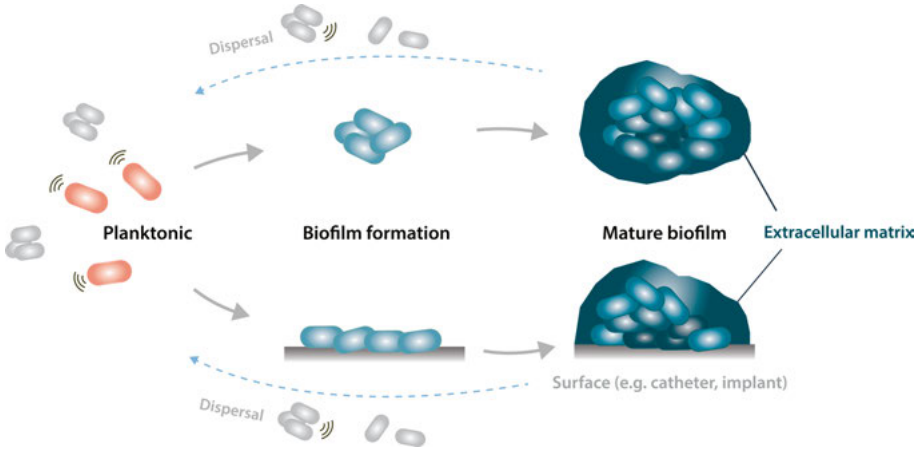

Figure 8 | Schematic illustration of biofilm formation. Planktonic bacteria attach to each other or to some surface and proceed to grow and produce extracellular matrix to form heterogeneous communities. Biofilm-dispersed cells can leave biofilms as single cells or aggregates.

Whatever kind of biofilm, it has to be initiated by the attachment, be it some kind of surface or other bacteria, when non-attached biofilms are formed (Figure 8). Surface organelles, such as fimbriae, extracellular polymers produced by the bacteria and present in the environment, can all affect the initiation of biofilm. Various mechanical forces, such as shear (hydrodynamic) forces, surface roughness or hydrophobicity, greatly influence how bacteria interact with surfaces (Persat et al., 2015; Hall Jr. et al., 2021). In **Paper I**, we also investigated the impact of different surfaces on the formation of *K. pneumoniae* and *E. coli* biofilms. Although somewhat counterintuitive, flow versus static conditions can actually increase the strength of binding to the surface. For instance, the tip adhesin FimH of type 1 fimbriae and MrkD of type 3 fimbriae (described in the section *Fimbriae*) changes to a high affinity conformation during flow conditions and mediates attachment due to so-called catch bonds (Yakovenko et al., 2008; Stahlhut et al., 2013). Bacteria are even able to sense surface stiffness and adjust the biofilm program accordingly, which makes a lot of sense considering the wide range of available surfaces even in the human body (from soft mucus to hard bones or teeth). In *P. aeruginosa*, such stiffness sensing was recently shown to be mediated via retractions of type IV pili (Koch et al., 2022). The aggregation behaviors are also dependent on, for example, different cell surface modifications. Azimi et al. showed how changes in LPS, altering surface hydrophobicity, led to distinct aggregation (stacked versus clumping) in a CF *in vitro* model (Azimi et al., 2021). For bacteria that

have flagella and are thus motile (e.g., *P. aeruginosa*, *E. coli*), interaction with the surface is a cue to lose this organelle and start a sessile lifestyle instead.

Once established on a surface (or in other medium, such as mucus supporting biofilm initiation), bacteria can grow. As the biofilm population grows, a few features emerge: i) spatial restriction, ii) varying access to nutrients and oxygen, which leads to iii) gradients of nutrients, oxygen, and consequently growth rates. All of this results in probably the most important feature of a biofilm, heterogeneity, where even a genetically clonal population does not show homogeneous phenotypical behavior (Stewart and Franklin, 2008). Biofilms in general switch to a completely different transcriptional program compared to planktonic ones, but especially as the biofilm matures, differences depending on where in the biofilm a bacterial cell is become more pronounced. The production of various ECM components encloses the biofilm community in a gel-like material, that can incorporate a diverse set of molecules. Water, both bound to other molecules and free, is a major component of a biofilm constituting as much as 90% of the total biofilm biomass, where long and narrow water channels have been proposed to serve for transport, and larger water pores for storage (Quan et al., 2021). A variety of polymers can be part of the ECM, such as exopolysaccharides, extracellular DNA, lipids and proteins (Whitchurch et al., 2002; Limoli et al., 2015; Sugimoto et al., 2018; Sigurlásdóttir et al., 2019; Karygianni et al., 2020), some of which are discussed in later sections. ECM is essentially what makes a biofilm a "biofilm". In **Papers III** and **IV**, we show that interfering with the production of specific ECM components either by deleting the corresponding synthesis genes (*pga*) or by enzymatic hydrolysis (e.g., cellulase), biofilms cannot reach a mature state in specific mutants.

Even when it looks like the biofilm is fully formed and there is nothing else to build so to say, it is never really a static existence, as recently reviewed (Katharios-Lanwermeyer and O'Toole, 2022). There is a tremendous amount of regulation, communication, and production going on in the biofilm communities just to "stay put" as the authors describe it, like in a multicellular organism. Dispersal represents a way for sessile bacteria to disseminate and establish in new places by partially breaking down the surrounding ECM and allowing cells to be released (McDougald et al., 2011). Although it may seem that dispersed bacteria are the same as planktonic ones, they seem to represent a distinct phase with specific transcriptional profile, as shown, for example, in *K. pneumoniae* (Guilhen et al., 2016). Dispersal and initiation can take form as aggregates instead of single cells (Kragh et al., 2016). Furthermore,, biofilm-dispersed *K. pneumoniae* was able to form microcolonies faster and attach to lung epithelial cells better (Guilhen et al., 2019), connecting the biofilm lifestyle with the actual infectious processes. Dispersal is essentially connected with the ability to cause systemic infection, as shown for *P. aeruginosa*, where enzymatic treatment dispersed the wound biofilm, but led to lethal systemic infection in mice when not coordinated properly with

antibiotics (Fleming and Rumbaugh, 2018). This illustrates an extremely important point that even if the biofilm is dispersed, antibiotic treatment has to be administered together to avoid systemic infection, which becomes difficult if the biofilm-forming bacteria are genetically MDR, like many *K. pneumoniae* strains. If the biofilm is not eradicated, it essentially keeps on serving as a source of bacteria that then can lead to recurrent infections and as a source of bacteria acquiring possibly adaptive mutations, as described in the beginning of this thesis.

Various environmental conditions affect different stages of biofilm formation, including early attachment, biofilm structure, or ECM composition. Some of the many factors that affect the biofilm formation are specific nutrients, such as nitrate (Martín-Rodríguez et al., 2020) or iron (Wu and Outten, 2009; Wu et al., 2012; Shen et al., 2017). Antibiotics, even at sub-MIC concentrations, can also induce biofilm (Song et al., 2016), for example, via ribosomal stress and connected c-di-GMP signaling, as shown for PNAG synthesis in *E. coli* (Boehm et al., 2009).

Biofilms and infection

In medical situations, it is beneficial for bacteria to form biofilms due to protection both from antibiotic treatments, exacerbating the antibiotic resistance problem, and the immune system (Ciofu et al., 2022). Multiple reasons can contribute to this protection. One of them is the above-mentioned physiological heterogeneity, which results in varying growth states of the cells and therefore varying susceptibility to antibiotics (Walters et al., 2003; Stewart et al., 2015). Certain antibiotics might have reduced diffusion due to the ECM or they can be bound by matrix components, therefore, not reaching the effective concentrations across the biofilm, although it is highly dependent on the antibiotic (Lebeaux et al., 2014). Similarly, matrix can provide protection from the immune effectors and phagocytic cells are unable to engulf the aggregates (Ciofu et al., 2022). While the immune system cannot eliminate the biofilm, it still elicits an immune response and consequently tissue damage due to chronic inflammation (Williams et al., 2010; Kolpen et al., 2022).

Medical devices were among the first to be connected with biofilm-related infections, for instance, *S. aureus* on a pacemaker (Marrie et al., 1982). Biofilms growing on medical devices have been shown to be much thicker (reaching up to 1 mm) than non-attached ones that are usually 100-200 μm in size (Bjarnsholt et al., 2013). In general, medical device surfaces, compared to biotic surfaces, are attractive due to the lack of host defense factors. However, the propensity for biofilm formation differs among different medical devices. Urinary catheters are extremely prone to becoming sites for biofilm formation, when compared, for instance, with intravascular catheters for several reasons, such as i) a high number of contaminating bacteria in the insertion site, ii) a difference in the fluid flow around the catheter (constant and fast blood flow vs intermittent flow of urine), and iii) changes in the bladder

microenvironment upon catheter insertion (Trautner and Darouiche, 2004). One such common change is release of fibrinogen, shown to be abundant on urinary catheters due to a locally induced inflammatory response upon catheter insertion in the bladder (Flores-Mireles et al., 2016), and this promotes biofilm formation in multiple bacteria, including *K. pneumoniae* (Flores-Mireles et al., 2014; Walker et al., 2017; Andersen et al., 2022). Interfering with fibrinogen deposition on catheters in a catheterized mouse model prevented CAUTI, illustrating the crucial role of this host protein (Andersen et al., 2022). Most of the patients who experienced infections during the hospital outbreak in **Paper II** had urinary catheters and developed UTIs. We also showed that some outbreak isolates with an increased biofilm capacity needed a fibrinogen coating to display the phenotype (**Paper II**). Furthermore, the maintenance of the experimentally evolving biofilm populations with fibrinogen was much more stable than on bare silicone (**Paper III**). In the best-case scenario, biofilm-infested devices can be removed, and when this is not possible (e.g., permanent implants), it can lead to devastating consequences, considering that antibiotic treatments are often not effective on biofilms (Macleod and Stickler, 2007; Singhai et al., 2012; Høiby et al., 2015; Percival et al., 2015). The advances in medicine make the use of temporary or permanent devices a new norm. Paradoxically, the same devices that enable the care of critically ill patients frequently become the source of serious infections. Therefore, extensive research is ongoing to modify medical device surfaces so that they resist biofilm growth by including antimicrobial agents, modifying the nanostructures of the surface, and other approaches (Costerton et al., 2005; Pavithra and Doble, 2008; Dickson et al., 2015; Palka et al., 2020; Hall Jr. et al., 2021). Infections involving biofilm growth are not limited to device-related infections though. Biofilms have been observed to contribute to periodontitis, lung infections, urinary tract infections and other infections (Bjarnsholt et al., 2011; Lebeaux et al., 2014; Høiby et al., 2015; Ciofu et al., 2022). Although typically thought to be related to chronic infections, a recent study also found biofilms in acute lung infections with bacteria in biofilms eliciting a weaker immune response (Kolpen et al., 2022). Biofilms have also been found in acute wounds (Schaber et al., 2007).

Biofilm models

> "It was necessary to experiment a little before a satisfactory technique for placing the slides in the water was developed." Arthur T. Henrici describing one of the first methods to study biofilms in Journal of Bacteriology, 1933.

The complexity and diversity of biofilms make it impossible to have one standard assay to study them, and the development of new models or assays to study biofilms go hand in hand with the research on biofilms. In the early

days, simple (although large in size!) devices were used to study marine bacteria, such as glass slides attached to an anchor (Henrici, 1933) or a somewhat more advanced but related concept with glass slides attached to a piece of wood and a lead carrier (Zobell and Allen, 1935). Although simple, such approaches let the researchers reach important conclusions, as Heinrici writes: "It is quite evident that for the most part the water bacteria are not free floating organisms, but grow upon submerged surfaces" (Henrici, 1933).

Although biofilms are abundant all around us, it is not easy to replicate the exact conditions to grow them *in vitro* (Roberts et al., 2015). Which leads to an ongoing debate in the biofilms field: should we have standardized assays so that we can compare the results from different studies easier and include them in clinical screenings, or should we treat biofilms as individual entities and proceed with complex and customized assays for each biofilm? A standard easy-to-use assay could help in clinical settings, but at the same time if the same assay does not give reliable results for all biofilm-forming bacteria (which is likely considering how diverse they are), what purpose does it serve? There have been attempts to have at least some kind of standardization, and currently the Calgary/MBEC device, the drip flow reactor, the CDC reactor, and a rotating disc reactor are considered standard methods for studying medical biofilms (Gomes et al., 2018). There are multiple ways to group numerous biofilm model systems as reviewed by many authors (Lebeaux et al., 2013; Roberts et al., 2015; Guzmán-Soto et al., 2021), and this section is not aimed at mentioning every device possible. They can be described in terms of i) *in vitro* vs *in vivo*, ii) static vs flow-based, and iii) surface-associated vs non-surface. The majority and still the most widely used are *in vitro* models. The origins of the commonly used microtiter plate model date back to (Fletcher, 1977) who studied bacterial adhesion to polystyrene, and then was further modified (Christensen et al., 1985; Mack et al., 1994; GA et al., 1999). Plates can also be coated, for example, with human extracellular matrix or collagen (Jagnow and Clegg, 2003). This simple setup allows primarily to study the attachment and is often accompanied by the crystal violet (CV) staining to quantify biofilm biomass. However, it is more difficult to use when the strains/species tested are poor biofilm formers, as the threshold for detection is rather high (Stiefel et al., 2016).

The main disadvantage of the microtiter plate model is that it is prone to sedimentation, thus minimizing the role of active attachment. It is also tricky to manipulate the biofilms, for example, to expose them to treatments or perform viable counts. In an attempt to address these issues, the Calgary device was created (Ceri et al., 1999). It is also based on a 96-well format, but the biofilms grow on pegs that protrude from a plate lid. In this case, the effect of sedimentation is minimized and it is much easier to transfer biofilms to fresh medium or expose them to antibiotics. The latter was basically why this device was created and later given the commercial MBEC™ device name (Ceri et al., 2001; Harrison et al., 2010) to depict its use for the determination of minimal

biofilm eradication concentration. However, practical issues also exist with this model, especially if one wants to disrupt the biofilm populations for downstream analysis, for example, viable cell counts or isolation of certain mutants, because that requires physically removing the plastic pegs from the lid with pliers. This can lead to unintentional biofilm disruption, contamination, and in the worst case – loss of a peg if it happens to just fly away during such procedure. In addition, this device is expensive, as it is only one time use and generates a lot of plastic waste. With the aim to keep high-throughput and biofilm formation on a peg but make it easier to work with, we designed the FlexiPeg device (**Paper I**) that is 3D printed, with pegs separately inserted, and thus easily removable from the lid, and re-usable (Figure 9). The main disadvantage of peg systems is that biofilms typically cannot be sampled or imaged during growth.

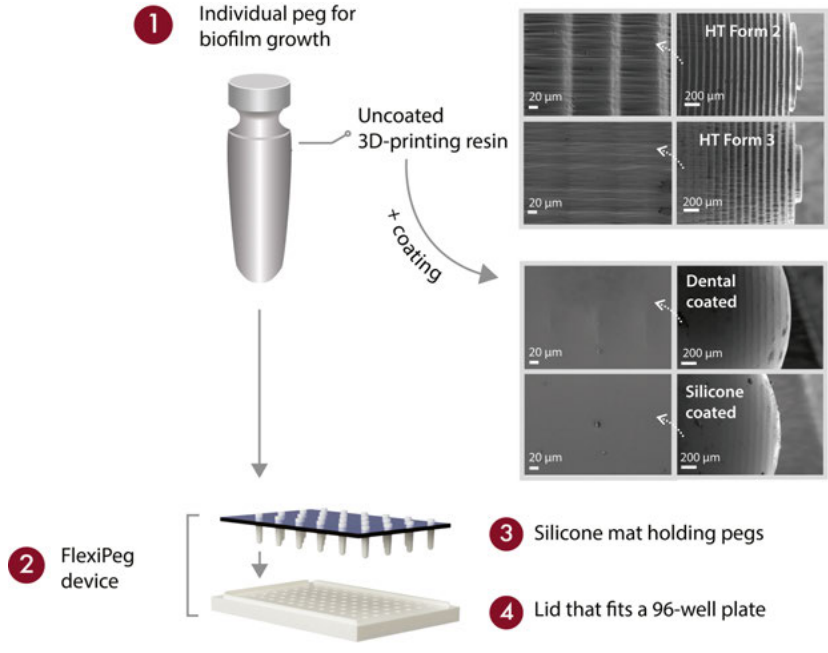

Figure 9 | An overview of the FlexiPeg device for the growth of surface-attached biofilms presented in Paper I. SEM images show the surface of different types of pegs. Form 2 and Form 3 refer to different 3D-printers that use different 3D-printing technologies.

Shear forces, as mentioned above, can affect binding strength (e.g., catch bonds by FimH and MrkD), but also the overall structure and strength of a biofilm (Stoodley et al., 1998; Persat et al., 2015). Therefore, the properties of a biofilm formed under static and flow conditions are likely to differ, as shown with antibiotic or disinfectant susceptibility (Buckingham-Meyer et al., 2007). Some of the classical models to study biofilm formation under flow-based

conditions include flow-cell reactors, where the flow cell with channels is covered with a glass slide and connected to fresh medium and waste via tubing, and the flow is created by a peristaltic pump. Typically, such biofilm setups are used together with confocal fluorescence microscopy to observe the biofilm *in situ* in real time, for example, when accompanied by fluorescent reporters (Zaborskyte et al., 2017), which is the greatest advantage of such systems. However, this setup is not suitable for downstream analysis of the whole biofilm population, is prone to contamination, bubble formation, and thus destroying the biofilms, and in general requires a lot of space and maintenance. Practically easier is the CDC reactor, where multiple coupons can be inserted into one container and the fluid flow maintained (Goeres et al., 2005). Recently, flow-based *in vitro* systems have been switching to microfluidic approaches (Kim et al., 2012; Tang et al., 2022). Such devices allow *in situ* biofilm observation by microscopy, like classical flow systems, but have much higher level of precision in terms of condition control. The downside is that typically, because of the small size, the experiments cannot be continued for a long time.

To mimic specific conditions present in the host, there has been extensive development in models that are either *in vitro* but incorporate some *in vivo* components or that are completely *in vivo* models (Guzmán-Soto et al., 2021). Some examples of *in vitro* models mimick CF lung environment with eDNA and mucin (Azimi et al., 2021). The flow-based 3D model for chronic wounds was designed to grow biofilms on a semipermeable membrane and with the flow rate more similar to the actual wound (Duckworth et al., 2018). Furthermore, analysis of the actual biofilms from the patients (*ex vivo* samples) can be informative to reveal the structures and interactions between different species. However, it is practically challenging and can only give a snapshot of the end-point without any information about how the process was initiated. In this case, animal models can serve as a proxy for biofilm-related infections in humans, especially when they are specialized for certain types of infections. For example, chronic wound models (Fleming and Rumbaugh, 2018; Vanderwoude et al., 2020), UTI and CAUTI models in mice are some of the most common (Walker et al., 2017; Martín-Rodríguez et al., 2020; Andersen et al., 2022), especially when they are accompanied by actual observations from patients and include clinical strains.

The results of biofilm experiments can be highly dependent on the biofilm model (Townsend et al., 2020). Therefore, when possible, it is best to combine multiple approaches to assess biofilm phenotypes. The results reporting whether some strains can or cannot form biofilms should always refer to the model with which it was assessed, as it can have a huge impact on the phenotype when the strain is not universally good at biofilm formation (generalists vs specialists). For example, Wzc mutants do not form surface-attached biofilms in a microtiter plate model, but they do form strong biofilms on silicone (**Paper III**). In contrast, some isolates from the outbreak clearly form biofilms

in various setups: aggregate in shaking cultures, attach to polystyrene and silicone (**Paper II**). In **Paper I** (Zaborskytė et al., 2022), we present a new easy-to-use 3D-printed device suitable for high-throughput biofilm studies on different surfaces, which we then used in various experimental setups, for example, screening in **Paper II**, experimental evolution and describing different biofilm phenotypes in **Papers III** and **IV**.

What about *K. pneumoniae* biofilms?

The recognition of biofilm formation in *K. pneumoniae* began long before the term "biofilm" was conceived. For example, Duguid writes in his observations from 1959 referring to *K. pneumoniae* lacking fimbriae: "They differed from the fimbriate strains in growing less abundantly and usually in failing to form a pellicle on broth." (Duguid, 1959). Later, fimbriae have been directly connected to the ability of *K. pneumoniae* to cause bladder and kidney infections in rats (Fader and Davis, 1980). It was also observed to attach to a glass surface *in vitro*, and this attachment was connected to increased tolerance to chlorine compounds (LeChevallier et al., 1988). Attachment to live tissues, namely bladder epithelial cells, as well as formation of aggregates in urine was demonstrated by scanning electron microscopy (SEM) (Reid et al., 1992). *K. pneumoniae* is common on urinary catheters alone or in mixed biofilms with other bacteria or fungi such as *Candida albicans* (Macleod and Stickler, 2007; Frank et al., 2009; Singhai et al., 2012; Vuotto et al., 2014). It is also reported to be common on other devices, for example, endotracheal tubes (Inglis et al., 1989). Like uropathogenic *E. coli* (UPEC), *K. pneumoniae* is also able to form intracellular biofilm-like aggregate communities (IBC), at least transiently (Rosen et al., 2008). It is not clear how colonization in the gut or nasal mucosa is related to biofilm formation in *K. pneumoniae*; however, in **Paper II**, we show that isolates with mutations leading to an increased biofilm phenotype show an advantage during GI competition in mice.

From a mechanistic point of view, biofilm research has been rather biased in terms of the covered bacterial species, where *Pseudomonas aeruginosa* is a clear winner. *E. coli* has been the most studied representative of the *Enterobacteriaceae*, and *K. pneumoniae* has been undeservingly left in the shadows as a "more encapsulated" cousin of *E. coli* when it comes to detailed biofilm formation analysis. Yet, despite all the similarities, there are already known fundamental differences between *E. coli* and *K. pneumoniae* biofilms, for example, *K. pneumoniae* is non-motile, lacks curli adhesins, and the role of cellulose is not established. Even for factors that are shared (e.g., type 1 fimbriae), *K. pneumoniae* has additional factors, like FimK (see section *Fimbriae*). Considering that the mechanistic side of biofilm formation in *K. pneumoniae* is underexplored, it is likely that many additional factors remain to be revealed. Like with virulence or colonization, there were attempts of global screens combined with transposon insertion libraries or signature-tagged

mutagenesis to identify genes important in biofilm formation in a microtiter model or flow-based systems with human extracellular matrix coating (Lavender et al., 2004; Boddicker et al., 2006; Wu et al., 2011). Some of the identified mutants unable to form biofilms pointed to the requirement for type 1 and type 3 fimbriae, the presence of K2 capsule, uncharacterized transcriptional regulators, and sugar phosphotransferases (Boddicker et al., 2006). However, these approaches suffer from a bias to loss-of-function changes. Analysis of fosmid libraries generated from *K. pneumoniae* C3091 and transduced in *E. coli* was connected to positive screening (i.e., increase in biofilm rather than decrease) in a microtiter plate model (Stahlhut et al., 2010). The results mainly confirmed the requirement for type 3 fimbriae, validating such an approach but not really expanding much on what other genes are important in *K. pneumoniae* biofilms. Considering the results from our studies, it is not that surprising that the presence or absence of a certain gene does not give much information, as many of the changes selected for increased biofilm formation in **Paper III** are not just a simple loss-of-function.

As mentioned above, *K. pneumoniae* C3091 (UTI) has been used in biofilm studies before to study the role of type 1 and type 3 fimbriae (Oelschlaeger and Tall, 1997; Struve et al., 2009; Schroll et al., 2010). IA565 with a unique plasmid-encoded cluster was also studied (Hornick et al., 1995; Langstraat et al., 2001; Childers et al., 2013), while the biofilm formation of the outbreak index isolate DA14734 has not been evaluated before. Although all of these three strains are capable of forming biofilms more or less depending on the surface, they are not even close to the strong biofilm phenotypes displayed by the outbreak isolates (**Paper II**). In **Paper III**, we experimentally evolved these three strains towards increased biofilm formation on three different surfaces. This thesis, especially **Paper III** and **Paper IV**, is aimed to expand the knowledge on *K. pneumoniae* biofilms with a particular focus on the role of different genetic variants. The following sections focus on specific factors or pathways important in biofilms with a special connection to what is known about *K. pneumoniae* biofilms.

c-di-GMP signaling

> ”Compared to the much-better-known cyclic nucleotide second messenger cAMP, c-di-GMP was about as popular as Kombucha tea compared to black or green tea. This all changed at the turn of this century <…> .” Römling et al., 2013, reviewing the first 25 years of research on c-di-GMP in *Microbiol Mol Biol Rev*.

Although biofilms are highly diverse, there are some factors that unify them. Intracellular secondary messenger bis-(3’-5’)-cyclic dimeric GMP (c-di-GMP) can be one of the examples, as increasing intracellular concentrations have been associated with biofilm formation, while decreased levels lead to

increased motility and dispersal (Simm et al., 2004). In the simplest terms, for such regulation to occur, a cell needs i) a way to increase and decrease the levels of the signaling molecule, ii) to couple such changes with sensory signals to integrate external and internal cues, and iii) to equip proteins with the ability to respond to such changes. So, how do bacteria achieve that in the case of c-di-GMP?

The question is not that straightforward to answer, as Römling et al. accurately refers to c-di-GMP signaling as a "regulatory nightmare" in their comprehensive review (Römling et al., 2013). However, it is so widespread that there is no option to simply ignore it. The synthesis and degradation of c-di-GMP is carried out by abundant proteins with some of the most conserved protein motifs, signifying the ubiquitous role of such proteins. The synthesis part is carried out by diguanylate cyclases (DGC) with a GGDEF domain (Gly-Gly-Asp-Glu-Phe) and hydrolysis to GTP by phosphodiesterases (PDE) with EAL (Glu-Ala-Leu) and/or HD-GYP domains (Ausmees et al., 2001; Paul et al., 2004; Simm et al., 2004; Schmidt et al., 2005). Typically, bacteria carry multiple EAL/GGDEF domain proteins (Galperin et al., 2001; Chou and Galperin, 2016), including on plasmids (Madsen et al., 2018). *K. pneumoniae* is not an exception and has an abundant presence of EAL/GGDEF domain proteins (Cruz et al., 2012), and our studied strains have multiple genes encoding EAL (at least 10) or GGDEF domain proteins (3-5) with various domain architectures, where each of the domains is present in the protein alone or, for instance, in combination with a sensory domain. Interestingly, Madsen et al. showed that the pUUH239.2 plasmid from the outbreak clone studied in this thesis, carries a GGDEF domain protein, and it was able to increase biofilm formation in other *Enterobacteriaceae* species upon transforming them with the cloned gene on a plasmid vector (Madsen et al., 2018). Proteins with GGDEF/EAL domains are not necessarily enzymatically active, for example, CsrD in *E. coli* (Suzuki et al., 2006), further complicating the analysis of these networks.

One of the best characterized effector protein domains is the PilZ domain, which binds c-di-GMP with high affinity (Schirmer and Jenal, 2009). This domain is also present in the MrkH transcriptional activator in *K. pneumoniae*. In fact, it was the first example where, through detailed structural analysis, the PilZ domain was demonstrated to directly mediate DNA binding (Schumacher and Zeng, 2016) and transcriptionally regulate the expression of type 3 fimbriae (Wilksch et al., 2011). Although there are many more domains responsible for responding to c-di-GMP, in general they are much more difficult to find than EAL/GGDEF proteins themselves due to their highly diverse sequences (Römling et al., 2013). Regulation by c-di-GMP can be achieved via regulation on multiple levels: transcriptional (e.g., type 3 fimbriae via MrkH, PNAG via DosC), post-transcriptional and post-translational (e.g., PNAG via PgaC-PgaD) (Römling et al., 2013; Chou and Galperin, 2016). In addition to

proteins, c-di-GMP can be sensed by riboswitches, therefore, further expanding the diversity of these signaling cascades (Sudarsan et al., 2008).

Since a single cell harbors so many different GGDEF and EAL domain proteins, the question arises about the specificity and redundancy of the signaling. Possible scenarios are illustrated in Figure 10. The simplest and most fitting the original "increase means biofilm, decrease means no biofilm" would be scenario A, where regardless of which enzymes perform the synthesis of c-di-GMP, a single effector mediates a response via a single target. In a more complicated version, specific enzymes interact with specific effectors, but they act on the same target. For instance, PNAG synthesis in *E. coli* is regulated by c-di-GMP at both the transcriptional and post-translational levels by two different DGCs. Transcription is induced by DosC (YddV) (Tagliabue et al., 2010) and the production of c-di-GMP by another DGC YdeH that stimulates PgaC-PgaD protein-protein interactions, and thus their glycosyltransferase activity (Steiner et al., 2013). Another variation of this scenario could be a sequential signaling cascade, where the synthesis of c-di-GMP by one DGC enzyme induces the production of another DGC, which then directly affects the target. The regulation of cellulose synthesis in *E. coli* serves as an example of such a cascade: synthesis of c-di-GMP by YdaM DGC activates the expression of the *csgDEF* operon, and CsgD induces the expression of another DGC enzyme, AdrA, which acts as an allosteric activator of the Bcs complex (Zogaj et al., 2001; Weber et al., 2006). In the most specialized scenario, each part of the signaling is separated either spatially (and thus mediated by smaller local changes in c-di-GMP levels) or temporally. For example, Merritt et al. showed that two diguanylate cyclases, SadC and RoeA, in *P. aeruginosa* did not result in a global change in c-di-GMP levels, but rather that different subcellular localization and thus local changes in c-di-GMP were driving the specific response (Merritt et al., 2010). Similarly, c-di-GMP signaling in *Vibrio cholerae* exhibits high specificity mediated by different PDE and DGC enzymes rather than a simple global change by each of them (Massie et al., 2012). A study of 28 different c-di-GMP turnover proteins in *E. coli* showed condition-dependent (e.g., temperature) expression (Sommerfeldt et al., 2009). Another recent study generated a library of 41 deletion mutants of individual PDE/DGCs in *P. aeruginosa* PAO1 and determined their contribution to initial attachment and overall biofilm structures (Eilers et al., 2022). The results pointed to the importance of local versus global changes, since clear and mutant-specific phenotypes were exerted even without global changes in c-di-GMP. They also cautioned against extrapolation of c-di-GMP signaling network data between different strains (Eilers et al., 2022). Experimentally evolved *P. aeruginosa* also showed varying phenotypes when mutations in c-di-GMP turnover proteins were detected, suggesting alterations in specific pathways (Wong et al., 2012; Flynn et al., 2016). Furthermore, since heterogeneity is an inherent feature of biofilms, c-di-GMP differences in individual cells or certain subpopulations in a biofilm might not be reflected in the

population level measurements (Nair et al., 2017). In **Paper IV**, we see global changes in c-di-GMP levels with highly induced toxin and immunity protein pointing in different directions, and multiple features (type 3 fimbriae, cellulose, PNAG) are regulated via special c-di-GMP dependent pathways, described in this section. It remains to be determined how this complex phenotype is orchestrated. In **Paper III**, we show that certain mutations (in *yfiR*, *yfiN*, and other putative diguanylate cyclases) can indeed alter global levels of c-di-GMP. However, other changes, even when the known c-di-GMP regulation is involved (e.g., MrkJ), do not necessarily induce such a global effect. Although diguanylate cyclase enzymes were affected, the most repeatedly selected mutations in connection to c-di-GMP were in phosphodiesterases, suggesting that it is easier to remove degradation than to increase the synthesis.

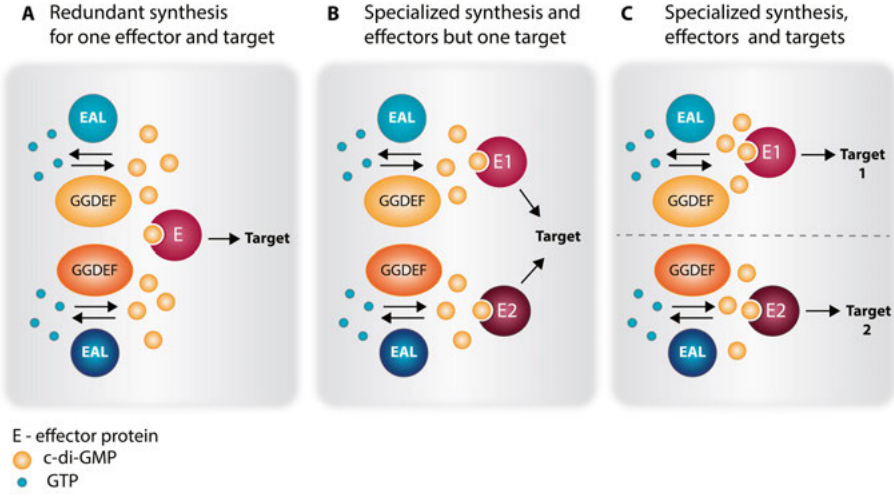

Figure 10 | Examples of c-di-GMP signaling pathways with differences in specificity of the responsible diguanylate cyclases (GGDEF domain), phosphodiesterases (EAL domain), effector proteins, and targets.

The input signals activating certain c-di-GMP pathways are relatively underexplored, but various factors have been identified so far, such as temperature (Sommerfeldt et al., 2009; Almblad et al., 2021), interaction with the epithelial surface (Raterman et al., 2013; Laventie et al., 2019) and envelope stress response, where *P. aeruginosa* activates diguanylate cyclase WspR after sensing, for instance, unfolded proteins in the periplasm (O'Neal et al., 2022). In relation to virulence, a fine-tuned regulation rather than a simple increase or decrease in c-di-GMP levels is important to ensure that the right components are made at the right time (Valentini and Filloux, 2019). This is illustrated by a study of the *yfiRNB* operon in *E. coli*, where inactivation of a negative regulator for the diguanylate cyclase YfiN leads to increased production of curli

fimbriae and cellulose, but this phenotype leads to complete attenuation in a murine UTI model (Raterman et al., 2013). The role of this operon in *K. pneumoniae* is discussed in more detail in the sections below, when talking about fimbriae and cellulose. Increased levels of c-di-GMP were also associated with attenuated virulence of *K. pneumoniae* in murine pneumonia model due to strong upregulation of type 1 and 3 fimbriae (Rosen et al., 2018), which are not beneficial in this niche, as also discussed later (section *Fimbriae*). Therefore, although initially low c-di-GMP levels were suggested to be the hallmark of acute infections, and high levels are typical for chronic infections, the regulation is more complex and includes many specialized pathogen- and niche-dependent responses as recently reviewed (Valentini and Filloux, 2019).

As with many biofilm-related factors, research in c-di-GMP turnover and signaling in *K. pneumoniae* is lagging behind, although for such a complicated regulatory network, it can be excused. While some DGC and PDE proteins have been characterized and assigned specific functions (e.g., the MrkJ phosphodiesterase and the YfiN diguanylate cyclase, described more in the next sections) (Johnson and Clegg, 2010; Huertas et al., 2014), many of them are still uncharacterized. The phosphodiesterase YjcC in hvKp was shown to be involved in a complex regulatory network connecting the oxidative stress response, CPS and type 3 fimbriae expression, and overall virulence in mice (Huang et al., 2013). However, the importance of c-di-GMP signaling is clear, and we see many of the c-di-GMP related genes under positive selection during the hospital outbreak (**Paper II**) and adaptive biofilm mutations accumulating during short-term experimental evolution in **Paper III**. One of the most interesting examples we discovered is a putative DGC with a sensory domain where the mutation A203V confers a wrinkly phenotype (unusual for *K. pneumoniae*) in the C3091 strain and where the same mutation appears in a UTI isolate from the hospital outbreak with a wrinkly phenotype as well.

Fimbriae

Various cell surface structures can mediate the essential part of biofilm formation, the attachment to each other or to surfaces. One of the examples for a species that frequently harbor conjugative plasmids, such as *K. pneumoniae*, is conjugative pili, which have been shown to promote adherence simply by establishing a close contact between the cells in *E. coli* (Ghigo, 2001). However, such non-specific interactions serve more as a way to bring bacteria closer and then initiate stronger interactions with the surface. Fimbrial adhesins represent probably the most characterized biofilm-related structures in *K. pneumoniae* with respect to clinical importance, evolutionary differences and, molecular regulation. The two major types, type 1 and type 3, are mannose-sensitive and mannose-resistant adhesins, respectively, produced by the chaperone-usher pathway (Nuccio and Bäumler, 2007; Zavyalov et al., 2010). In this case, chaperones make sure that the fimbrial subunits do not self-

aggregate during assembly and present them to the usher protein that is located in the outer membrane and mediates the correct assembly on the cell surface. In *K. pneumoniae*, type 1 fimbriae are important in UTI (Fader and Davis, 1980; Struve et al., 2008), but type 3 fimbriae are essential in the formation of biofilms on abiotic surfaces, reflecting the possible difference between CAUTI (Schroll et al., 2010; Murphy et al., 2013); however, their roles depend greatly on the exact niche. Other less explored fimbriae/pili also exist, for example, those homologous to *E. coli* common pilus (ECP) (Alcántar-Curiel et al., 2013) and others (Khater et al., 2015).

Type 1 fimbriae

Mannose-sensitive type 1 fimbriae are encoded in the *fim* cluster (Figure 11). Its expression is regulated by phase switching: recombinases FimB and FimE mediate the inversion of the invertible element *fimS* enabling expression of the structural genes downstream, where FimB can turn the switch in both directions, while FimE is more prone for the ON to OFF switch (McClain et al., 1991). In addition to these genes, the *fim* cluster encodes FimA (main fimbrial subunit), FimC (chaperone), FimD (usher protein), and three proteins for the tip: the minor subunits FimF and FimG, and the main tip adhesin FimH, which recognizes mannosylated receptors (Schwan, 2011).

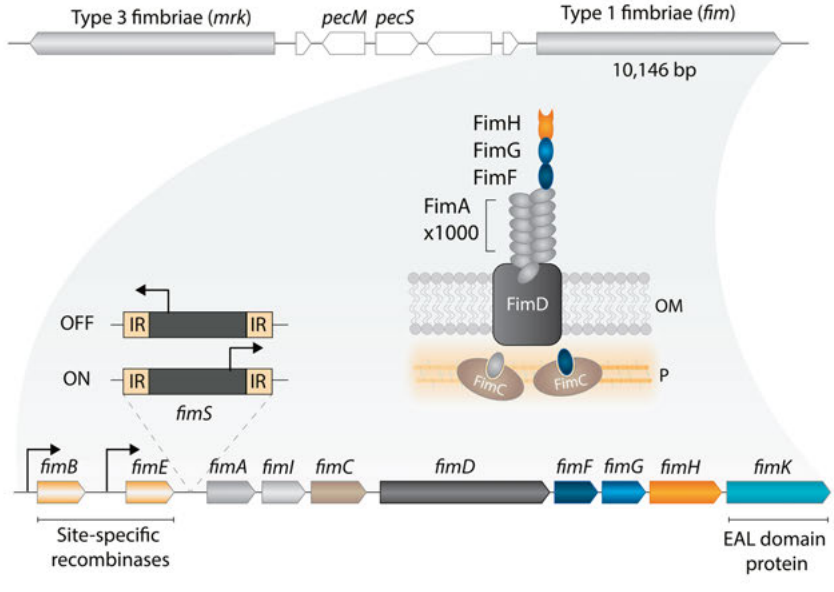

Figure 11 | Type 1 fimbriae cluster in *K. pneumoniae*. The expression of *fim* genes is regulated by phase switching mediated by recombinases.

Although type 1 fimbriae in *K. pneumoniae* are homologous to those in *E. coli*, the amino acid sequence similarity varies from 72 to 84 % (Struve et al., 2008). These differences translate into different sugar binding and attachment to epithelial cells, illustrating species-specific differences. Additionally, *K. pneumoniae* carries a unique additional gene *fimK* that negatively affects the *fim* switch, thus most of the time keeping *fim* in the OFF position, in contrast to UPEC (Gerlach et al., 1989; Rosen et al., 2008). In contrast to UTI, FimK increases *K. pneumoniae* virulence in pneumonia (mice), reflecting niche-specific regulation and consistent with differential requirements for fimbriae in these environments (Rosen et al., 2016). Interestingly, the expression of *K. pneumoniae fimK* from a plasmid vector in UPEC also decreased biofilm formation, suggesting an involvement in a conserved regulation pathway (Rosen et al., 2008). Since FimK has an EAL domain, it was suggested to be involved in c-di-GMP alterations, and thus possibly link the regulation of type 1 and type 3 fimbriae in *K. pneumoniae* (Wang et al., 2013), and currently the exact role is being elucidated. The *fim* clusters in C3091 and IA565 are basically identical with slight differences in length, but DA14734 seems to have some additional *fimA* genes in multiple genomic locations, suggesting some recombination events, similar to the capsule locus in this strain. We observed point mutations in *fimD* (usher) and *fimH* (tip adhesin) in outbreak isolates (DA14734 clone), and the latter were present in isolates with increased biofilm formation, as well as in an isolate showing better GI colonization in mice (**Paper II**). Pathoadaptive mutations in the *fimH* of UPEC and *K. pneumoniae* from UTI were also found (Sokurenko et al., 1998; Stahlhut et al., 2009). Mutations in *fimI* (unknown function) and *fimD* were also selected during experimental evolution, but only in the DA14734 strain (**Paper III**), hinting at more diversification in this strain.

Type 1 fimbriae are critical to the pathogenesis of UTI in mice or rats (Fader and Davis, 1980; Struve et al., 2008). Consistent with that, injections of D-mannose prevented the attachment of *K. pneumoniae* and subsequent infection in rats (Fader and Davis, 1980). Preventing attachment by specifically targeting type 1 fimbriae is one of the main strategies in drug discovery against UTIs (Pinkner et al., 2006). In *K. pneumoniae* (C3091, one of our studied UTI strains), the ON or OFF status depends on the niche inside the host: ON in the urinary tract, but OFF in the lungs and during intestinal colonization in mice (Struve et al., 2008). Even within the urinary tract, the need to have an ON or OFF switch depends on the exact location, as shown for *E. coli*: while it is advantageous to express these fimbriae in the bladder with abundant mannose receptors, it is switched off in the kidneys to avoid detection by the immune system (Schwan, 2011).

Type 3 fimbriae

Type 3 fimbriae in different *Klebsiella* strains and their adhesive properties were first described a long time ago (Duguid, 1959), although not really

showing what they are made of. Later, they were found to be thin (2-4 nm wide) filamentous protein adhesins, encoded by the *mrk* (mannose-resistant *Klebsiella*-like) gene cluster located upstream of the *fim* cluster, described above (Gerlach et al., 1989; Allen et al., 1991; Struve et al., 2009). As the name implies, unlike type 1 fimbriae, type 3 fimbriae do not bind mannosylated receptors and the exact receptor is still not clear. The *mrk* cluster encodes i) the structural proteins MrkA (major fimbrial subunit), MrkF (stability, minor subunit) and MrkD (tip adhesin), ii) the assembly proteins MrkB (periplasmic chaperone) and MrkC (usher protein), and iii) the regulatory proteins MrkH (transcriptional regulator dependent on c-di-GMP), MrkI (LuxR-type transcriptional regulator, function not clear) and MrkJ (phosphodiesterase) (Figure 12). The genes encoding for these proteins are organized into three transcriptional units: polycistronic operon *mrkABCDF*, bicistronic *mrkHI* and *mrkJ*. Type 3 fimbriae are important for the formation of biofilms on abiotic surfaces, such as silicone catheters, but also for attachment to host extracellular matrix components, like collagen (Jagnow and Clegg, 2003; Murphy et al., 2013; Stahlhut et al., 2013). While typically MrkD mediates the attachment, on some abiotic surfaces MrkA can be enough (Langstraat et al., 2001). MrkF has been shown to be intermittently incorporated together with MrkA, in contrast to minor subunits from type 1 fimbriae, where they only assemble at the very end (Huang et al., 2009). It has also been shown to affect the length of type 3 fimbriae.

For a long time, type 3 fimbriae have been associated with *Klebsiella* strains only, where they were first found; however, now there are reports describing *mrk* clusters on conjugative plasmids (e.g., pOLA52) that have been transferred to, for instance, *E. coli* and enhance biofilm formation in this species (Burmølle et al., 2008). IA565 strain also carries an additional plasmid-encoded *mrk* cluster that differs from the chromosomal one (Hornick et al., 1995). They have also been found in other species involved in CAUTI (Ong et al., 2010), suggesting frequent horizontal transfer events. However, while in *K. pneumoniae* type 3 fimbriae seem to be nearly universal across different strains, a study screening over 700 *K. pneumoniae* and *E. coli* isolates found that only approximately 2% of *E. coli* carry them in contrast to 95% of *K. pneumoniae* (Stahlhut et al., 2013). Comparison of *mrkD*, encoding the tip adhesin, alleles in *K. pneumoniae* and *E. coli* strains suggested that those in *K. pneumoniae* are more diverse, evolutionary ancient and under purifying selection, while those in *E. coli* were relatively recent, displayed less effective binding, and showed signs of adaptive evolution (Stahlhut et al., 2013). MrkD, harbored by the UTI isolate C3091, one of the strains studied here, represents the most abundant allele and exhibits the strongest binding to yeast mannan (Stahlhut et al., 2013). Like FimH, for which the structure is known, MrkD is predicted to have fimbriae-anchoring pilin domain (185-332 a.a.) and lectin domain (24-184 a.a.) that converts to high-affinity conformation and mediates formation of catch bonds (Stahlhut et al., 2013), as mentioned earlier (section

General biofilm features). MrkD, or more specifically lectin domain of this protein, was one of the shared targets during experimental evolution in **Paper III**, with an in-frame deletion of G40-V43 selected in all three strains along with some other point mutations or bigger structural variations. In addition to better attachment to silicone, deletion of G40-V43 increased the attachment to lung epithelial cells in IA565 strain.

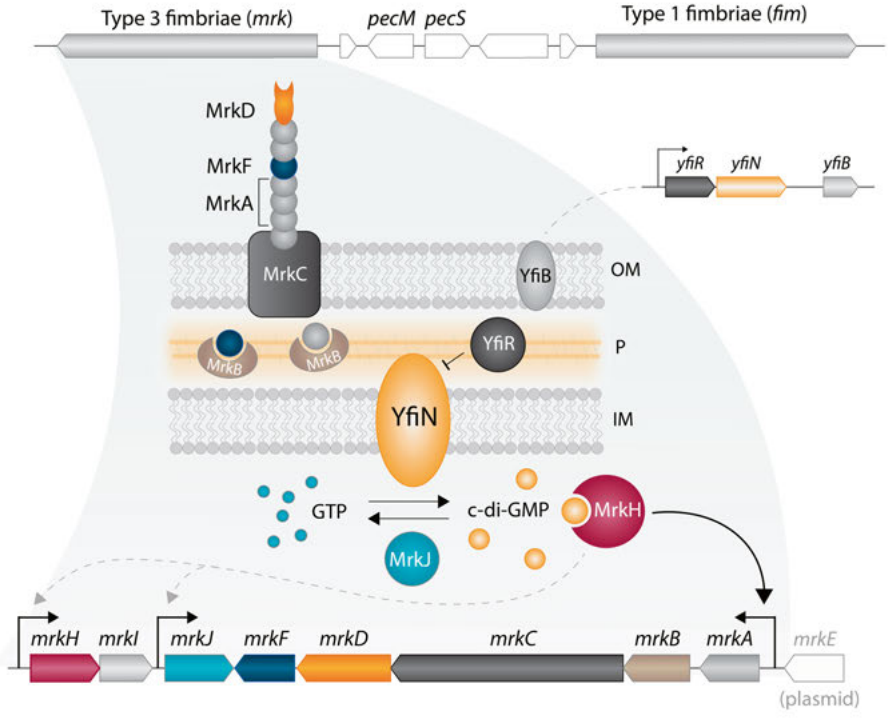

Figure 12 | Type 3 fimbriae cluster and its regulation in *K. pneumoniae*. The expression of *mrk* genes is dependent on c-di-GMP.

Regulation of type 3 fimbriae expression is dependent on c-di-GMP through MrkJ phosphodiesterase, YfiN diguanylate cyclase, and the PilZ domain transcriptional regulator MrkH (Johnson and Clegg, 2010; Huertas et al., 2014; Tan et al., 2015; Schumacher and Zeng, 2016; Ares et al., 2017), as already mentioned in the previous section (section *c-di-GMP signaling*). MrkH activates the expression of *mrkABCDF*, *mrkJ* and itself (Wilksch et al., 2011; Schumacher and Zeng, 2016; Ares et al., 2017); however, its regulatory role seems to be complex, for example, excess MrkH can repress *mrk* genes (Ares et al., 2017) and it also antagonizes the effects of global regulators like H-NS that upregulates the expression of *mrkA*, but represses the expression of *mrkJ* in a temperature-dependent manner (Ares et al., 2016, 2017). Iron availability also affects fimbrial expression via Fur and IscR (Wu et al., 2012; Shen et al.,

2017) and it is likely that additional regulators will be revealed in the future. For example, recently, a putative EII complex (EtcABC) was shown to be involved in the activation of *mrk* via cAMP (Panjaitan et al., 2019).

MrkJ phosphodiesterase was one of the main targets in our experimental biofilm evolution (**Paper III**). Additionally, YfiN and YfiR mutations were selected in the C3091 strain and we show that mutations in MrkJ, YfiN and YfiR affect *mrkA* expression in a strain, mutation and growth state dependent manner (**Paper III**). We also show that the combination of capsule loss with an increase in fimbriation via inactivation of MrkJ leads to an extreme aggregation phenotype (**Paper III**). The biofilm phenotype of the Spyo^{T361S} mutant includes upregulation of both type 1 and type 3 fimbriae (**Paper IV**).

Capsule and extracellular polysaccharides

Polysaccharides are abundant components of the ECM, which is essential for biofilms (Flemming and Wingender, 2010; Limoli et al., 2015). Various roles have been assigned to polysaccharides, such as protecting the biofilm from desiccation, providing a scaffold for other components, or mediating adhesion (Flemming et al., 2016). In contrast to other bacteria (e.g., alginate, Pel, Psl in *P. aeruginosa*, cellulose, PNAG in *E. coli*), the composition of exopolysaccharides in *K. pneumoniae* biofilms is not that clear and is dependent on the strain (Benincasa et al., 2016; Bandeira et al., 2017) as many other biofilm-related features discussed above.

Capsule is abundant in many *K. pneumoniae* strains, as discussed in an earlier section on capsule synthesis, however, the role of it in biofilms is not straightforward. Some studies have shown directly that capsule is part of the ECM, for example, the capsular K24 polysaccharide was detected in *K. pneumoniae* biofilms by structural NMR analysis (Cescutti et al., 2016). Two other *K. pneumoniae* strains, one with good adhesion and the other with lots of ECM and non-adhering, were investigated with respect to their exopolysaccharide structures, revealing completely different structures that resembled CPS, but the authors did not specify whether these exopolysaccharides were the same as CPS in these strains (Benincasa et al., 2016). Mutations in *treC* (trehalose-6-phosphate hydrolase) and *sugE* decreased or increased, respectively, CPS production and biofilm formation in a microtiter model, pointing to a direct positive correlation between capsule and biofilm (Wu et al., 2011). However, there are observations of a negative effect of CPS on certain types or specific stages of biofilm formation. Capsule can shield bacterial adhesins, thereby interfering with initial attachment (Schembri et al., 2004). The deletion of capsular genes was shown to increase biofilm formation (Huang et al., 2014). *K. pneumoniae*, like other Gram-negative bacteria, can form pellicles at the air-liquid interface (Armitano et al., 2014; Chabane et al., 2014), and hypermucoviscous K1 *K. pneumoniae* formed thick pellicles but could not attach to polystyrene surfaces (Cubero et al., 2019). Ernst et al. showed that loss-of-

capsule WbaP mutants in ST258 formed better surface-attached biofilms than hypermucoid Wzc mutants in a microtiter plate model. In **Paper III**, we show that both increased capsule phenotype (via Wzc mutations) and loss of capsule can lead to strong biofilm phenotypes, but the strength of the phenotype is dependent on the strain and surface versus non-surface attached biofilm. In **Paper II**, we also show that the same mutation (deletion in YrfF) conferring an increase in capsule can increase biofilm formation, but hypermucoid isolates with mutations in addition to Wzc were not good at forming silicone-attached biofilms. Thus, overall, it looks like capsule involvement is dependent on multiple factors, including the strain (and KL type), the biofilm model/surface, the stage of the biofilm, and specific mutations affecting capsule production.

Poly-β-1,6-N-acetyl-D-glucosamine (PNAG) is one of the most common bacterial exopolysaccharides, and its disruption has been shown to negatively affect biofilm formation by various bacteria, such as *E. coli*, *P. aeruginosa*, *Staphylococcus epidermidis,* and *Yersinia pestis* (Itoh et al., 2005). In many bacteria, such as *E. coli,* the synthesis and export of PNAG is encoded by the *pgaABCD* operon (Wang et al., 2004). The synthesis of the polysaccharide is carried out by PgaC glycosyltransferase that interacts with PgaD (the exact function is unclear) in the presence of c-di-GMP, and the polysaccharide is transported via PgaA porin while simultaneously being deacetylated by PgaB (Whitfield et al., 2020). In *K. pneumoniae*, there are some reports on the effects of inactivating certain genes in the *pga* operon. For example, Wu et al. generated a *pgaA* transposon mutant of hvKp with decreased biofilm formation (Wu et al., 2011). Chen et al., showed that in-frame deletion of *pgaC* reduces *in vitro* biofilm formation in the presence of bile salts and negatively affects GI colonization and systemic spread of *K. pneumoniae* CG43 in mice (Chen et al., 2014), connecting PNAG with biofilm formation and with invasive liver abscesses. In **Paper IV**, we show that the PNAG polysaccharide specifically contributes to the *K. pneumoniae* Spyo^{T361S} mutant biofilm.

Cellulose synthesis, which is common in biofilms in many bacterial pathogens, such as *E. coli* and *Salmonella*, is encoded by the *bcs* operon; however, the exact organization of the *bcs* operons varies a lot (Römling and Galperin, 2015). Cellulose production was the first process shown to be directly regulated by the c-di-GMP messenger in a fruit-rotting bacterium *Gluconacetobacter* (previously *Acetobacter*) *xylinus* (Ross et al., 1987). As mentioned above (section *c-di-GMP signaling*), cellulose synthesis in *E. coli* is regulated by c-di-GMP in a complex sequential signaling cascade (Zogaj et al., 2001; Weber et al., 2006). The specific diguanylate cyclase YdaM activates the transcription of the *csgDEFG* operon, which is responsible for the production of curli fimbriae. The CsgD protein then activates the transcription of *adrA*, encoding another diguanylate cyclase that allosterically activates the Bcs machinery through BcsA. However, the expression of cellulose can differ even in bacteria that carry genes for its synthesis (constitutive in *E. coli*, but

inducible in some other bacteria, for example); therefore, other ways might exist (Römling et al., 2013). BcsB (non-catalytic subunit in the periplasm bound to the inner membrane) interacts with BcsA (catalytic subunit) to produce the growing polymer chain that is then transported through the BcsC translocon (Povolotsky and Hengge, 2012; McNamara et al., 2015). Data on the role of cellulose production in *Klebsiella* biofilms are scarce, but *K. pneumoniae* strains, including all three strains studied in this thesis, carry *bcs* genes, and there are published indications of cellulose synthesis via YfiN and YfiR (Zogaj et al., 2003; Huertas et al., 2014). Therefore, it could be that YfiN serves as a connecting point between regulation of cellulose and type 3 fimbriae in *K. pneumoniae*. In fact, we observed that the YfiN and YfiR mutants affected both the type 3 fimbrial expression and the production of cellulose (**Paper III**). The biofilms of two of the strains C3091 and DA14734 are at least partly sensitive to cellulase enzyme, hinting at some cellulose production. In **Papers III** and **IV**, we touch upon the role of cellulose in biofilms formed by the evolved mutants and show through enzymatic hydrolysis and transcriptional changes that specific point mutations in c-di-GMP turnover proteins (YfiR, YfiN, MrkJ) or direct cellulose synthesis genes (e.g., BcsB, BcsZ) upregulate cellulose production in evolved mutants.

Bacterial interactions

As presented in the previous sections, bacteria do not exist as solitary cells: they form biofilms and compete with the microbiota to establish themselves in the sites of colonization. Therefore, interactions, both positive and negative, are a key feature in the lives of bacteria. Microbial interactions have also been studied by applying an experimental evolution approach (Martin et al., 2016a). Communication between bacteria can be achieved in multiple ways which can be roughly divided into secretion of certain molecules (e.g., soluble bacteriocins, quorum-sensing molecules) and contact-dependent interactions (e.g., R/F pyocins, type V or type VI secretion systems). In connection with **Paper IV**, this section is focused on bacteriocins and type VI secretion systems.

Bacteriocins

Bacteriocins represent proteins with narrow-spectrum antimicrobial activity affecting closely related species or strains. The first bacteriocin, colicin, was identified in 1925 (Gratia, 1925), although not really called that back then, and then further explored to reveal that colicin production is induced by the SOS response and that colicin-producing strains are protected from its toxicity because of the immunity protein (Jacob et al., 1952). Bacteriocins have historically been named after the bacterium that produces them, such as colicins from *E. coli* or pyocins from *P. aeruginosa*; however, there is quite a lot of confusion in their nomenclature (Cascales et al., 2007). Bacteriocins from Gram-negative bacteria can be divided into colicins, colicin-like bacteriocins

(klebicins, S-pyocins), phage-tail-like (R- and F-pyocins) and the smallest ones (<10 kDa) – microcins that are produced by enterobacteria (Baquero and Moreno, 1984). Genes for producing microcin (*mce*) are also present in the IA565 strain studied in this thesis, possibly adding to its good colonization ability, as observed in the murine pneumonia model, where this strain was rapidly cleared from the lungs, but could easily colonize nasal mucosa for extended periods of time (Lau et al., 2007b, 2008). Klebicins produced by *K. pneumoniae* have also been described, but there are only a few studies presenting those with nuclease activity and recent pore-forming and peptidoglycan-degrading toxins (Ghequire et al., 2018; Denkovskienė et al., 2019).

Bacteriocins enter the cells in a receptor-dependent manner and they exploit the essential components of the bacterial cell surface for this process, employing a "Trojan horse strategy". As mentioned above (section *Iron acquisition and metabolism*), the siderophore receptors but also outer membrane porins, such as OmpC often serve as entry points for bacteriocins. Like colicins (e.g., colicin M), klebicins also require the ferrichrome receptor FhuA or OmpC to get into the cell and TonB with ExhB for translocation (Riley and Wertz, 2002; Braun, 2009; Ghequire et al., 2018; Denkovskienė et al., 2019). Resistance to bacteriocins has been shown to arise more frequently under iron-rich conditions, probably reflecting the dispensable use of siderophores in these conditions (Inglis et al., 2016). In **Paper II**, we hypothesize whether loss-of-function mutations in FhuA could have been selected as protection from bacteriocins, especially since they were also combined with a mutation in SbmA, serving as a transporter for microcins.

Type VI secretion systems (T6SS)

First observed in nodule-forming plant bacterium *Rhizobium leguminosarum* (Bladergroen et al., 2003), T6SS-associated genes were soon connected to pathogenesis in a fish and human-infecting *Edwardsiella tarda* (Srinivasa Rao et al., 2004), virulence of *P. aeruginosa* (Mougous et al., 2006) and *V. cholerae* (Pukatzki et al., 2006). Abundant studies since then have mostly placed T6SS as an interbacterial weapon that is widespread in many commensal and pathogenic bacteria (Boyer et al., 2009). Even the same strain can have multiple different T6SS with a diverse set of functions, for instance, some specialized for virulence with the effect on eukaryotic cells and others for interbacterial competition as shown in *Burkholderia* (Schwarz et al., 2010). *K. pneumoniae* has been shown to harbor T6SS, but there have been very few studies on this so far (Liu et al., 2017; Hsieh et al., 2019; Storey et al., 2020).

T6SS represents a complex machinery that spans the membranes and is made of at least 13 different components. From structural, functional, and evolutionary perspectives, T6SS is homologous to contractile bacteriophage tails (Basler et al., 2012). The main parts (Figure 13) include i) the membrane complex (TssJ, TssL, TssM proteins), ii) the baseplate in the cytoplasm (TssE, TssF, TssG, TssK) that initiates the assembly of iii) contractile sheath (TssB,

TssC) and the inner tail tube (Hcp) and iv) the spike (VgrG, PAAR domain proteins and different effectors) that physically pierces the cell surface. TssH (ClpV) ATPase mediates the recycling of sheath subunits for another attack (Gallegos-Monterrosa and Coulthurst, 2021). The diversity of T6SS functions is dictated by numerous effector proteins whose targets range from DNA, RNA, peptidoglycan, phospholipids, and pore formation (Hernandez et al., 2020; Jurėnas and Journet, 2020). The effector proteins can be translated as separate proteins that are then loaded onto the T6SS spike via specific interactions (cargo effectors), or they can be translationally fused with one of the components, such as VgrG (valine-glycine repeat) or Hcp (hemolysin coregulated protein) (Jurėnas and Journet, 2020). For example, Hcp-ET modules, which are effectors translationally fused with Hcp, have been shown to be abundant in *Enterobacteriaceae* (Ma et al., 2017). Typically, toxic effectors have their cognate immunity proteins (antitoxins) to avoid intoxicating the producing cell unless the target is a eukaryotic cell as well as protect themselves from neighboring cells (Yang et al., 2018). However, immunity-independent protection mechanisms also exist. For example, capsule has been shown to act as an immunity-independent protection for *K. pneumoniae* against T6SS attacks in the gut microbiota (Flaugnatti et al., 2021). The novel T6SS effector that we characterize in **Paper IV** is a DNase toxin with an S-pyocin-like domain. S-pyocin domain is also translationally fused with Hcp protein in the uropathogenic specific protein (Usp) of *E. coli* (Nipič et al., 2013). Recently, it was demonstrated that the PAAR domain Rhs toxin was not delivered but expressed inside the cell instead and that it regulated growth of *S. enterica* in macrophages (Stårsta et al., 2020), further expanding the functional diversity of T6SS-associated effectors. Interestingly, contact-independent killing by a T6SS effector acting as a DNase has also recently been reported (Song et al., 2021). In addition to toxic effects, DNase toxins associated with T6SS can have potentially adaptive roles towards the target cells by acting as mutagens (de Moraes et al., 2021).

T6SS is a costly machinery to make, therefore, it has to be tightly regulated. Different signals can regulate T6SS expression, for example, temperature, iron levels, osmolarity, or pH (Ishikawa et al., 2012; Storey et al., 2020). While there is a large number of studies identifying new effectors, the natural functions of T6SS are rather underexplored. It is not difficult to imagine that competitive advantage conferred by T6SS could be important in polymicrobial biofilms that are common on, for example, urinary catheters (Azevedo et al., 2016). However, it looks like T6SS can potentially have more diverse roles in bacterial lives, such as, signaling or community restructuring (Russell et al., 2014). T6SS-dependent regulation of the expression of type 1 fimbriae has been shown in avian *E. coli* (De Pace et al., 2010). Specific diguanylate cyclase has also been connected with inducing T6SS in *P. aeruginosa* (Moscoso et al., 2011). The Spyo^{T361S} mutant studied in **Paper IV** drastically increases biofilm formation in *K. pneumoniae* when expressed internally via

c-di-GMP dependent processes, illustrating a connection with cellular functions beyond toxicity. More T6SS-associated components (e.g., TssM (also called VasK), TssE, TssJ) were mutated during experimental evolution and in outbreak isolates (**Papers II** and **III**), illustrating involvement of such systems in *K. pneumoniae* lifestyle.

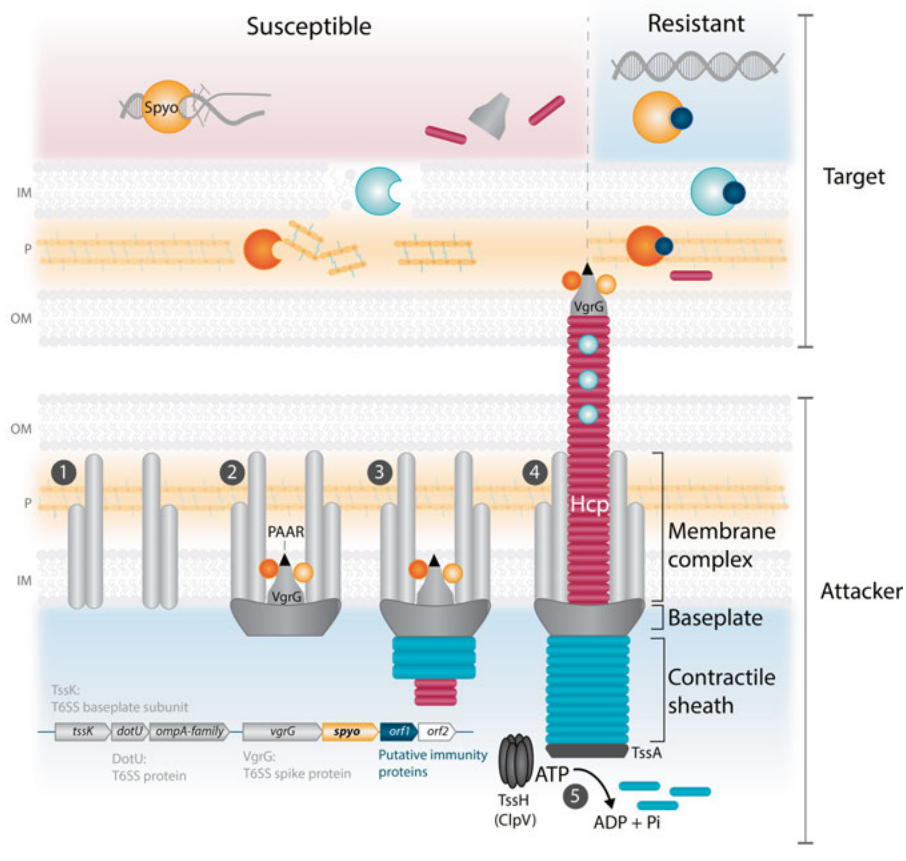

Figure 13 | Schematic overview of the T6SS machinery. (1) The membrane complex is assembled, attracting (2) the baseplate onto which the assembly of the spike with effector proteins and (3) the sheath and inner tail tube occurs. (4) Sheath contraction results in delivery of effectors into the target cell. The VgrG and Hcp subunits are also secreted. (5) TssH ATPase depolymerizes the sheath to prepare for the next attack. The genomic location of the Spyo toxin, studied in Paper IV, is shown in the lower left corner.

Current investigations

As presented in the introduction, *K. pneumoniae* is a pathogen of critical importance; however, we still lack essential information about its interaction with the host, including growth of biofilms, and especially how naturally selected mutations affect these phenotypes. Papers included in this thesis cover methods to make it easier to study biofilms, large-scale real-life evolution analysis, experimental evolution in a biofilm, and detailed molecular mechanisms of specific novel mutations. The following summaries introduce the key points for the rationale, methodology, and results of each study.

Paper I: a method to study biofilms

The diversity of biofilms requires equally diverse model systems to study biofilms in the lab. Quite often, though, one has to compromise between high-throughput and ease of use of the system. While it is true that the model should be as physiologically relevant as possible, the usability and options to adapt the same model to different experimental setups are equally important. Biofilms attached to abiotic surfaces are reminiscent of medical device-related biofilms, which are extremely common and associated with infections (Costerton et al., 2005; Koseoglu et al., 2006; Percival et al., 2015; Andersen et al., 2022). The Calgary device, or later commercially renamed as the MBEC^{TM} device (Ceri et al., 1999, 2001; Harrison et al., 2010), is one of the common simple assays for studying surface-attached biofilms, mostly due to its high-throughput nature. However, it is practically rather challenging to use due to a design where the pegs are permanently attached to the lid, especially if one wants to disrupt the biofilm for downstream applications, such as viable cell counts or mutant isolation. In addition, this device is single-use and expensive, even more so if additional surface modifications are desired.

To keep the high-throughput option of MBEC^{TM}, but to make it more versatile and easier-to-use, we designed a 3D-printed *in vitro* device to grow surface-attached biofilms. The device comes in two separate parts: the lid that fits a 96-well plate and the pegs that can be inserted into the lid in desired numbers and locations. The pegs can be made of different materials compatible with 3D printing as well as further coated afterwards, for example, with silicone. We named the device FlexiPeg to illustrate the flexible nature of this device

in terms of surface choice, the number of biofilms to be grown, and the experimental questions to be answered. All parts of the FlexiPeg device are re-usable after autoclaving, and we have not observed any deterioration in device performance after >30 cycles of repeated autoclaving-use cycles.

We validated the use of FlexiPeg with two species often associated with device-related infections, *E. coli* (CFT073) and *K. pneumoniae* (C3091 and DA69557). By employing viable cell counts after vortexing pegs to disrupt biofilms, crystal violet staining, and SEM imaging of intact biofilms, we confirmed that the device allows differentiation on a species and strain level. In addition, we showed the dependence of biofilm phenotypes, from early attachment to maturation, on the peg surface material. While the device is static, we argue that there is a considerable overlap between our observations and those from flow-based systems as well as clinical observations, for example, on catheters from real patients. The versatility and adaptability to different approaches is illustrated by the fact that we used this device for subsequent **Papers II**, **III** and **IV** for screening, experimental evolution, and fundamental biofilm experiments.

Paper II: evolution of *K. pneumoniae* during a hospital outbreak

During colonization and infection, bacteria encounter numerous challenges, such as immune defense, varying nutrient availability and antibiotic treatments, all of which can result in selection of certain genetic variants in bacterial populations. Short generation times mean that bacterial pathogens can evolve and adapt even during life in a single host and, depending on the body site where the mutant arises as well as associated trade-offs, can either be transmitted to other hosts or represent specific niche adaptations. Opportunistic pathogens, such as cKp, often spread in hospitals giving rise to outbreaks. Clonal outbreaks in particular present a unique opportunity to study within-host changes with a great resolution as opposed to studies of unrelated clones. The evolution of *K. pneumoniae* during outbreaks has been studied to a varying scale and extent (Snitkin et al., 2012; Gu et al., 2018; Marsh et al., 2019; Wylie et al., 2019), but most often it does not concern the same clone and is typically confined to genomic analysis.

An MDR clone of *K. pneumoniae* with the pUUH239.2 plasmid was the culprit for an outbreak at Uppsala University Hospital between 2005 and 2010 (Lytsy et al., 2008; Sandegren et al., 2012). More than 300 patients were affected, and they were typical targets for cKp: mostly geriatric, with many underlying diseases and receiving multiple antibiotic treatments. Such an outbreak presented a unique opportunity to look into how much a single bacterial clone changed during five years of colonizing and infecting the patients.

Therefore, we set out to characterize both genomic and phenotypic changes to gain insights into the important selective pressures driving within-host evolution of this clone.

The phylogenetic analysis of 110 isolates generated a tree with rather few clusters and mostly long individual branches, suggesting that most of the genetic changes occurred within individual patients in contrast to continuous spread of bacteria with certain changes. The genetic changes were dominated by nonsynonymous mutations in chromosomal protein coding genes, and the dN/dS ratios of genes with more than 3 independent genetic changes were strikingly high (>23). Therefore, there was clearly a positive selection for changes involving capsule and LPS synthesis (*manB, manC, wzc, wcaJ, wcoZ, cpsACP)*, outer membrane (*ompK36*), signal transduction via two-component systems (*barA, uvrY, cpxA)*, iron acquisition and metabolism (*fhuA, fepA, sufB, sufC)*, c-di-GMP dependent regulation (*yfiN, pleD/ycdT*), carbohydrate metabolism (*citA, scrR, exuR)* and transport *(sbmA, yehY)*. Furthermore, there were amplifications, deletions and point mutations on the pUUH239.2 plasmid. Analyzing overall groups of more than 500 unique genetic changes pointed to connection with antibiotic resistance, biofilm formation and virulence, thus, we decided to test them experimentally.

One obvious selective trait could be antibiotic resistance, which is a clearly testable phenotype and can be potentially connected with the antibiotic treatment. To get a systematic picture of antibiotic-mediated selection, we collected data on all antibiotic treatments each patient received during one year before confirmed to carry the clone. The results of such analysis showed many antibiotic treatments for individual patients (median 5, range 0-19), mostly with beta-lactams and fluoroquinolones. Phenotypically, we observed sporadic changes in resistance profiles, mostly in isolates from infection sites, with some clustering in terms of decreased susceptibility to piperacillin/tazobactam and an unexpected increased susceptibility to nitrofurantoin or macrolides. The most common changes were to beta-lactam antibiotics, and they were in many cases linked to IS26-mediated amplifications of different parts of the pUUH239.2 resistance cassette or loss-of-function OmpK36 porin changes. Since antibiotic resistance mutations typically can be transmitted much easier than host niche adaptations, antibiotic treatment in one patient can lead to transmission of resistant mutant to another one that had not been treated, making it more difficult to make direct connections as opposed to before and after analysis in a single patient. However, we could relate decreased piperacillin/tazobactam and carbapenem susceptibility to treatments of individual patients before isolation of the clone.

Since most infecting isolates were from UTIs, we focused the biofilm screening on a medical device-related setup, and stained biofilms grown on silicone-coated pegs in the FlexiPeg device (**Paper I**). In addition, we identified that certain isolates required fibrinogen to display the biofilm phenotype. In general, there were not many isolates differing (n=13), but those that had

increased the biofilm capacity, did so enormously. Most were not phylogenetically close, and in the cases where they were clustering, they acquired different mutations, supporting individual adaptations; however, the mutational targets overlapped pointing to convergent evolution via changes in type 1 fimbriae (FimH), c-di-GMP dependent regulation, for example, type 3 fimbrial regulation (MrkJ, MrkH, YfiN, YfiR, putative DGC), and regulatory capsule/cell envelope changes (YrfF, UvrY). The fact that most of the changes occurred in isolates from infections suggested short-lived adaptive changes.

To assess their potential in acute virulence, we tested all isolates in serum killing assays and the *G. mellonella* larvae infection model that has been increasingly used to decipher virulence properties in many bacteria, including *K. pneumoniae* (Insua et al., 2013; Wand et al., 2013; Bruchmann et al., 2021). Defects or increase in serum survival were mostly connected to cell surface structures, such as capsule and LPS. Interestingly, point mutations in the Wzc tyrosine autokinase led to a hypermucoid phenotype, also recently observed by others (Ernst et al., 2020). However, unexpectedly such isolates were extremely sensitive to human serum, raising the question about the mechanism behind such capsule change. Around half of all isolates were attenuated in *G. mellonella* larvae to some extent. By comparing phylogenetically close isolates with different virulence phenotypes, we could pinpoint the exact genetic change responsible for the virulence defect, for example, point mutations in ManB, ManC, or RfaH, or increase in virulence, for example, changes in GsiB, CsrD, and OxyR. However, the large number of genetic changes in many isolates made it quite difficult to draw strong conclusions without further genetic reconstructions. Changes in genes related to iron acquisition and metabolism were among the most common ones, therefore, we tested for growth in low iron conditions to assess any defects that could be responsible for virulence changes. We did not really find any clear cases of decreased growth in iron depleted conditions. Instead, there were two isolates with a mutation in *sufB* that seemed to grow better, and both of them showed increased killing in *G. mellonella*. Collectively, virulence analysis revealed extensive genetic diversification that phenotypically converged mainly to attenuation in systemic virulence.

Considering that infection by cKp is usually preceded by colonization (Martin et al., 2016b), we investigated colonization of the gastrointestinal tract in mice by isolates with different phenotypic profiles. Since the outbreak affected >300 people, the question arises whether the clone has certain factors that would make it a better colonizer in general. Thus, we were interested to find i) how well the clone can colonize GI with the intact microflora; ii) whether antibiotic-treated mice would be more prone to being colonized by the clone, and iii) whether any of the acquired mutations/phenotypes would provide a competitive advantage during the colonization with or without antibiotic treatment. To be able to resolve subtle differences, we performed head-to-head competitions with pairs of strains where the index isolate was tagged

(aminoglycoside resistance) to be able to separate them, instead of performing individual inoculations. We found that the clone was not really a "super-colonizer", and none of the tested isolates could be stably maintained before antibiotic treatment even with high inoculums. Interestingly, the bacterial count in faeces in all competition groups sky-rocketed as soon as mice started getting clindamycin in the drinking water, suggesting that numerous antibiotic treatments in patients during the outbreak could have facilitated the transmission of the clone. Additionally, all three isolates with increased biofilm capacity showed a competitive advantage, especially the isolate with a 21nt deletion in *yrfF* leading to increased capsule, while the hypermucoid isolate with a Wzc point mutation was worse at colonizing relative to the index isolate.

In summary, we show how a single *K. pneumoniae* clone has changed during within-host evolution in multiple patients in terms of diverse phenotypes connected to survival in the host. This study illustrates the complexity of real-life bacterial evolutionary trajectories even in a single clone and the need to explore both genetic and phenotypic changes as they can be coupled in ways that are hard to predict with each type of analysis alone.

Paper III: experimental evolution towards increased biofilm

The ability to form biofilms, especially on indwelling medical devices, is one of the major facilitating factors for *K. pneumoniae* infection. The protected bacterial community can withstand antibiotic treatments, immune attacks, and serve as a reservoir of infecting bacteria. Therefore, the increased capacity to form biofilms can be a pathoadaptive change directly related to better survival in the host.

Various biofilm-related genes and pathways have been studied in *K. pneumoniae* to different extent. Some of them, like type 1 and type 3 fimbriae, have been analyzed comprehensively from evolutionary, molecular, and clinical perspectives (Jagnow and Clegg, 2003; Stahlhut et al., 2009; Ong et al., 2010; Wilksch et al., 2011). There are limited studies on capsule and other extracellular polysaccharides, such as PNAG and cellulose (Zogaj et al., 2003; Chen et al., 2014; Huertas et al., 2014). Several large-scale transposon mutant library- or fosmid library-mediated screens have been reported, but mostly confirming the need for already identified genes (Lavender et al., 2004; Boddicker et al., 2006; Stahlhut et al., 2010; Wu et al., 2011). Therefore, the network of genes and different genetic variants that mediate biofilm formation in *K. pneumoniae* remains incomplete, while it is clear that biofilm formation can evolve within-host (**Paper II**).

Experimental evolution can be a powerful tool to identify adaptive changes without imposing a bias towards loss-of-function changes (Elena and Lenski,

2003) in contrast to the above-mentioned screens. In this study, we wanted to find out how three different clinical *K. pneumoniae* strains would evolve the increased biofilm capacity: how fast would it take, how similar would they be, and how much overlap would exist with the actual isolates from a clinical outbreak. To achieve this, we performed a short-term serial passaging in the FlexiPeg device (**Paper I**) with HT, silicone and silicone with fibrinogen coating, to connect to medical device surfaces (silicone) and host proteins facilitating biofilm formation on them (Andersen et al., 2022).

In this setup, the pegs were inoculated with a number of independent lineages (total of 38 for BHI, 12 for urine) from each strain and allowed to grow for 48 h. Then the populations were harvested by vortexing and i) transferred to a new plate with new pegs, ii) plated for viable cell counts and morphotype assessment, and iii) frozen for later analysis. Depending on the conditions, the cycling was continued for 5-10 cycles, as we could see rapid changes in overall biofilm morphology, morphotype dynamics, and viable cell count changes even after a few cycles in many lineages. To identify the genetic basis for these changes, we whole-genome sequenced the selected clones and some populations. The results gave a clear indication of convergent evolution, where the same targets (capsule, type 3 fimbriae, c-di-GMP-regulated processes) and even the same genes (*wzc, mrkD*, *mrkJ*) or protein positions were mutated across different strains and independent lineages. These targets, and in some cases, even the same mutations overlapped with the changes observed during the clinical outbreak (**Paper II**), suggesting that biofilm formation can be a selected pathoadaptive change inside the host.

We then further explored the phenotypes of the most common mutant groups. While they all were selected due to better biofilm formation, their phenotypes depended on the surface and the exact mutation. For example, point mutations in Wzc that led to a hypermucoid phenotype seemed to help cover the surface of the peg in two of the strains, while capsule loss led to the formation of more isolated aggregates as visible from SEM imaging. The hypermucoid mutant cells were also imaged by TEM to analyze the cell surface. While we could see increased production of capsular polysaccharides, it was clear that they were more likely to be shed away from the cells and that *wzc* mutation led to distortions in the cell envelope. Considering many changes in genes connected with c-di-GMP-dependent type 3 fimbriae regulation and possibly cellulose production, we explored the effects on *mrkA* and *bcsA* expression, biofilm treatments with cellulase enzyme and quantified global c-di-GMP levels. We show that fimbrial expression mutants are also connected to cellulose production via c-di-GMP-dependent regulation, something that has not been well established in *K. pneumoniae*. Importantly, we show that single mutations in the same gene (e.g., MrkJ) can alter the expression of fimbrial and cellulose genes differently and in a strain-dependent manner. In addition, some selected mutants had changes in uncharacterized EAL/GGDEF domain

proteins, one of which led to wrinkly phenotype, was sensitive to cellulase treatment and showed increase in global c-di-GMP levels.

In connection with survival inside the host, we also assessed some other infection-related phenotypes: survival in human serum, attachment to lung and bladder epithelial cells, and killing of *G. mellonella* larvae. Complement-mediated killing in human serum echoed the complex role of mutational capsule changes observed in **Paper II**: while capsule-loss mutants were killed as expected, the *wzc* mutation also led to extreme serum sensitivity. Interestingly, MrkD ΔG40-V43 in the IA565 background increased survival in serum. The same mutation increased the attachment to lung epithelial cells, again only in this strain, pointing to strain-dependent differences even for identical genetic changes. The survival of *G. mellonella* larvae in most cases was not affected; however, a RcsD histidine phosphotransferase mutant increased killing in IA565, Rho S325T led to attenuation in DA14734 and Wzc P642T in C3091 also decreased killing, illustrating that the relationship between biofilm formation and acute/systemic virulence is dependent on the exact genetic change underlying the biofilm phenotype.

In summary, we show how three *K. pneumoniae* strains are "a mutation away" from a different lifestyle: they rapidly acquire point mutations that are selected due to increased biofilm capacity but mediate a variety of diverse phenotypes relevant when considering how *K. pneumoniae* can evolve within-host.

Paper IV: regulation of biofilm formation by a T6SS effector

Communication is essential in bacterial lives and can be achieved both by secreted compounds and contact-dependent interactions. Type VI secretion system (T6SS) represents a fascinating contact-dependent machinery that functions mostly as an interbacterial weapon. T6SS is evolutionarily, structurally and functionally homologous to contractile phage that functions as a needle directly puncturing the target cell (Basler et al., 2012). The "poisonous tip" of such needles can be loaded with a cocktail of diverse effector proteins, such as those hydrolyzing nucleic acids, peptidoglycan, or forming pores in the membrane (Hernandez et al., 2020; Jurėnas and Journet, 2020). In **Paper III**, I repeatedly isolated the same mutation T361S in a gene encoding protein (Spyo) containing an S-pyocin domain in the IA565 strain. On closer inspection, it became clear that this protein is likely to be a T6SS effector, as its gene was located in one of the T6SS clusters of this strain, and right downstream *vgrG*, forming the spike of the needle. Here, we explore a peculiar mechanism where a toxic protein regulates biofilm formation, with a dramatic change by a single and specific nucleotide change leading to a T361S mutation.

To confirm that *spyo* indeed encodes a toxic protein, we cloned it on a plasmid vector and transformed into *E. coli* and *K. pneumoniae* cells together with putative immunity protein genes *orf1* or *orf2*. Induction of Spyo^{WT} led to an almost 3-log decrease in CFU within 30 minutes and *orf1 (spyoI)*, located downstream the *spyo* gene, rescued the cells, confirming that it encodes a cognate immunity protein. The T361S mutation clearly reduced the toxicity, but did not abolish it completely. Considering that S-pyocins typically have endonuclease activity (Michel-Briand and Baysse, 2002), we stained bacterial cells with fluorescent DAPI after toxin induction and could see signs of DNA damage. Transcriptomics analysis of *K. pneumoniae* with induced *spyo*^{WT} or *spyo*^{T361S} clearly showed upregulation of genes associated with DNA repair and SOS response, which was especially strong in the *spyo*^{WT} background already after 5 min of induction, confirming that Spyo is a DNase toxin and that T361S reduces its toxicity to some extent. The BLASTn search revealed that *spyo* in the same genomic organization is widespread in thousands of *K. pneumoniae* genomes, however, without the T361S mutation, suggesting that toxic activity is needed when living, for example, in polymicrobial biofilms or competing with gut microbiota.

The question is how is the toxic activity coupled to a phenotype that is on the opposite end of death – biofilm formation? We showed that the expression of type 3 fimbriae and the PNAG polysaccharide operon was increased in Spyo^{T361S} 6- and 8-fold, respectively. Interestingly, it was also higher in Δ*spyo*, which does not show any increase in biofilm, suggesting that some toxic activity is needed to display the phenotype. Additionally, in-frame deletion of *pgaA* prevented Spyo^{T361S} biofilm maturation, showing that this polysaccharide is important for biofilm phenotype and is specifically upregulated in the mutant. Spyo^{T361S} biofilms were also extremely sensitive to cellulase treatment, specifically hydrolyzing cellulose that can be part of ECM. We also looked at whether the biofilm phenotype is dependent on toxin delivery. While *vasK*, the essential component of the T6SS membrane complex, was expressed both in wt and mutant, deletion of this gene did not affect biofilm formation. Furthermore, growth of the wt strain together with T361S did not increase its biofilm formation, suggesting that the biofilm phenotype is not dependent on toxin delivery.

Exploring the *spyo* operon in more detail, we realized that the nucleotide affected by the mutation sits 50 nt downstream of predicted internal promoter, which prompted us to investigate the possible effect on transcription of immunity and a shorter version of the toxin. The promoter was indeed functional as it resulted in expression of YFP upon cloning on a plasmid vector. Neither toxin nor immunity expression was affected in the Spyo^{T361S} mutant, but Δ*spyo* showed a 50% reduction in *spyoI* expression. Connecting to the observation that the expression of biofilm-associated *mrkA* and *pgaA* was increased in Δ*spyo*, where we know *spyoI* is not expressed as much as in wt, we hypothesized that the immunity protein could be negatively affecting biofilm

formation. Therefore, we measured biofilm formation after transforming *spyoI* on low-copy and high-copy plasmid vectors into wt, Spyo^{T361S} and Δ*spyo*. In line with our expectations, overexpression of *spyoI* decreased biofilm formation, however, in Spyo^{T361S} the effect was only seen with the high-copy vector.

Immunity overexpression experiments pointed to the need for the right balance between toxin and immunity proteins, suggesting that this is something that could be changed because of the mutation. As we did not see any changes in toxin or immunity expression in the mutant, we decided to test protein stability. Quite surprisingly, it revealed that unlike type II TA systems, this SpyoSpyoI toxin-immunity pair has reversed protein stability features: the immunity protein is stable, but the toxin is not, and the T361S mutation made the toxin protein more stable. The fact that the mutation increases the stability of the toxin, means that the toxin can stay in the cell for longer, and thus it needs to be bound to immunity to prevent the death of the cell. This sequesters the immunity protein, which, as discussed above, would otherwise negatively affect the biofilm.

The above-described experiments showed that both toxin and immunity have effects on biofilm formation, therefore, we wanted to explore it in more detail. Since we saw changes in *mrkA* expression in Spyo^{T361S}, we measured the expression when toxins were induced from the plasmid vectors with and without SpyoI. SpyoWT alone, but not Spyo^{T361S} increased *mrkA* expression after 30 min induction, and both toxins with SpyoI increased it too. Since *mrkA* expression is regulated by the MrkJ phosphodiesterase and YfiN diguanylate cyclase, we repeated the immunity overexpression experiments in the loss-of-function MrkJ mutants (constructed or evolved in **Paper III**). There was no decrease in biofilm formation, showing that immunity protein could be acting through c-di-GMP regulation. We then measured the cellular concentrations of c-di-GMP by LC-MS in cultures with induced toxins with or without immunity. Consistent with biofilm decrease, SpyoI alone decreased c-di-GMP levels, but both wt and mutant toxins lead to a high increase in c-di-GMP. This suggests that i) the global c-di-GMP change is not a consequence of the T361S mutation; and ii) that this global change is most likely a response to something else that is directly regulated by the toxin. However, it is important to note that we only saw these global c-di-GMP changes with induced toxin, and not in the original T361S mutant where both toxin and immunity are expressed chromosomally.

We then turned to transcriptomics to get any insights on possible targets. As already mentioned, Spyo^{WT} showed upregulation of numerous genes related to DNA repair and SOS response, and in general there was very little transcriptional overlap between Spyo^{WT} and Spyo^{T361S}. Regarding biofilm related genes, we saw differential expression dependent on the background: i) both toxins showed upregulation of some type 3 fimbrial genes, ii) only toxins together with immunity could increase expression of cellulose synthesis (*bcs*)

genes, while Spyo^{WT} decreased it, and iii) only Spyo^{T361S} with immunity highly upregulated type 1 fimbriae genes. In addition, a putative phosphodiesterase was downregulated in Spyo^{WT}, but another uncharacterized diguanylate cyclase was instead upregulated in Spyo^{T361S}. This illustrates that c-di-GMP changes in wt and mutant could be achieved by different means and therefore lead to different downstream biofilm responses in agreement with, for example, highly specialized cellulose or PNAG regulation in *E. coli* (Zogaj et al., 2001; Weber et al., 2006; Tagliabue et al., 2010; Steiner et al., 2013).

In summary, we characterize a new T6SS effector with DNase activity in *K. pneumoniae* involved in biofilm regulation, showing that the functions of T6SS effectors extend beyond toxicity and can mediate important phenotypes even when not delivered.

Future perspectives

The work presented in my thesis paves the way for a number of possible investigations, especially the large datasets from **Paper II** and **Paper III**.

The method presented in **Paper I** is of a modular nature and 3D-printed, therefore, it presents many opportunities to adapt the setup. For example, the pegs can be printed in or coated with different materials, adjusted for experimental setups with larger wells, and possibly adapted to flow-based biofilm growth.

Paper II resulted in a large and unique dataset that combined both genotypic and phenotypic characteristics of a single *K. pneumoniae* clone that had undergone individual within-host evolution. Each isolate essentially serves as an individual track-record of interesting within-host evolution trajectories. It is obvious that numerous genes were under strong positive selection, and for some of the selected genetic variants, we could connect the genotype to phenotypic changes (increased biofilm formation, capsule loss or gain, some virulence changes). Biofilm phenotypes could be explained relatively easily narrowing down to one or two responsible mutations. However, the explanation for changes in virulence (serum survival and especially more complex *G. mellonella* killing) is not as straightforward for some isolates with a large number of combined mutations. Therefore, genetic reconstructions of specific changes could potentially help in those cases. The future work could be focused either at isolate level, that is explaining combination of changes in one isolate, or specific pathways affected across different isolates. Furthermore, considering how many diverse factors affect the survival of the pathogens inside the host, testing for other specific phenotypes (e.g., attachment to cells, survival in macrophages, resistance to bile salts) could reveal other interesting changes related to colonization/infection. Colonization experiments with additional isolates could help identifying the potential for certain within-host changes to be transmitted further.

Possible future work based on **Paper III** can be divided into experimental setup changes and a more detailed characterization of already selected mutants. The experimental setup could be altered, for example, by extending the cycling for longer, or skipping the dispersal stage to investigate how that affects the mutational profile and speed of selection. To find new pathways, we could start the cycling with strains lacking certain structures, for example, capsule or fimbriae. Additionally, since we do see some bias towards changes

selected in different strains, we could include additional strains, in particular hypervirulent *K. pneumoniae* strains or globally prevalent strains like ST258 that could potentially reveal additional evolutionary trajectories. The same kind of setup could be used to evolve *K. pneumoniae* in the presence of antibiotics, and therefore combine both selection for biofilm growth and antibiotic resistance in a single experiment. The already selected mutants represent a variety of interesting phenotypes, some of which were explored to some extent in this study. Wzc mutants is one the obvious examples and we would like to find out the exact mechanism of how these mutations mediate the phenotype, for example, by structural analysis of capsular polysaccharides (NMR). Another interesting uncharacterized protein is a GGDEF domain protein with sensory domain, in which mutation A203V confers a wrinkly phenotype, which is unusual in *K. pneumoniae,* but we also found the same mutation that confers the same phenotype in the outbreak isolate. We showed that it is associated with increased c-di-GMP levels and cellulose synthesis; however, the mechanism remains unclear and this would be an interesting thing to follow up. The global terminator Rho mutation resulted in an extremely strong phenotype and can potentially reveal new regulatory mechanisms. Another study related to c-di-GMP would be to explore the interplay between c-di-GMP turnover, cellulose synthesis and type 3 fimbriae in MrkJ, YfiN, YfiR, BcsB and BcsZ mutants, as we show that the responses in these pathways seem to be highly specialized. Mutants can also be investigated in more detail or in terms of different phenotypes. For example, analysis of survival in macrophages is currently ongoing to complement the virulence profiles. Also, analysis of how the biofilms formed by mutants affect tolerance to antibiotics has been initiated in our group.

Paper IV, in which we have identified a new T6SS-associated toxin, serves as an initiation point for further characterization. While we have already shown that Spyo is indeed a toxin with DNase activity and that the mutation T361S changes the stability of the toxin, the exact mechanisms remain to be investigated. We are currently performing chromatin immunoprecipitation sequencing (ChIP-Seq) to identify possible DNA binding sites of the toxin and the immunity protein. This could help to identify the exact regulatory pathway that connects them to biofilm formation. We have shown that unstable Spyo becomes more stable upon acquiring the T361S mutation and the increased biofilm phenotype suggests altered levels of free immunity in the cell, therefore, hinting at an altered interaction between toxin and immunity. To investigate how the mutation affects the toxin-immunity complex, we are working on protein pull-down assays. So far, our data point to the delivery-independent mediation of the biofilm phenotype in the Spyo^{T361S} mutant. However, we have been trying to determine whether Spyo can be delivered by T6SS in general, which is likely based on the genomic location directly downstream of *vgrG*, encoding the spike of T6SS. Usually, T6SS are costly to express, and they become active only in specific conditions. We have conducted interbacterial

competitions under various conditions, but so far could not show T6SS-dependent delivery. This is likely due to an IS element inserted in one of the *vasK* genes, encoding a part of the essential membrane complex. We are currently working to clone Spyo into another *K. pneumoniae* strain with similar but active T6SS and testing whether we can detect delivery there. In addition, we also want to perform co-immunoprecipitation with Hcp and VgrG, which are secreted into the extracellular space upon sheath contraction. This would show direct involvement of Spyo in T6SS-mediated secretion. Finally, the transcriptomics data are the most recent addition to this study and the complete analysis of the numerous detected changes is still ongoing. We also have preliminary data that the Spyo mutation could be affecting (increasing) virulence, as tested in *G. mellonella*, therefore, it could be explored further.

Populiarioji santrauka lietuvių kalba

Kaip evoliucionuoja *K. pneumoniae* bakterijos: nuo eksperimentinės bioplėvelių evoliucijos iki protrūkio ligoninėje

Žmogaus organizme gausu įvairiausių veiksnių ir iššūkių, kuriuos tenka įveikti čia patekusioms bakterijoms: imuninės sistemos komponentai, kintančios sąlygos skirtinguose audiniuose ir organuose ar konkurencija su kitomis bakterijomis, pavyzdžiui, žarnyne. Dauguma bakterijų dauginasi greitai, o tai reiškia, kad kiekvienos replikacijos metu atsiranda daugybė genetinių ”klaidų“ (mutacijų), kurios gali turėti įvairų poveikį skirtingoms savybėms pasireikšti, pavyzdžiui, kaip bakterija geba apsiginti nuo imuninės sistemos ar įsitvirtinti tam tikruose audiniuose. Priklausomai nuo mutacijos poveikio ir konkrečių aplinkos veiksnių, natūralioji atranka lemia skirtingų genetinių variacijų įsitvirtinimą, o tuo pačiu bakterijų prisitaikymą prie tam tikros nišos, pavyzdžiui, šlapimo ar kvėpavimo takų.

Klebsiella pneumoniae bakterijos yra vienos svarbiausių žmogaus patogenų, ypač klinikinėje aplinkoje. Sparčiai augantis atsparumas antibiotikams bei itin greitas prisitaikymas prie įvairių nišų leidžia šioms bakterijoms sėkmingai plisti ir sukelti sunkiai gydomas infekcijas, pavyzdžiui, šlapimo takų infekcijas, plaučių uždegimus, pooperacinių žaizdų užkrėtimus. Pasaulio Sveikatos Organizacija yra priskyrusi šias bakterijas prie kritinės svarbos patogenų, kuriems kuo skubiau reikalingos naujos gydymo galimybės. Šioje disertacijoje pristatau tyrimus, kaip *K. pneumoniae* bakterijos evoliucionuoja protrūkių ligoninėse metu bei laboratorinėje aplinkoje, pavyzdžiui, formuojant bioplėveles ant silikoninių paviršių. Tyrimų rezultatai reikšmingai prisideda prie fundamentalaus *K. pneumoniae* pažinimo, kuris būtinas norint sėkmingiau kovoti su šiomis bakterijomis, o taip pat prie bendro supratimo apie tai, kaip bakterijos prisitaiko infekcijų metu.

Didelė darbo dalis buvo skirta *K. pneumoniae* bioplėvelių tyrimui. Bioplėvelės apibūdina specifinę bakterijų augimo būseną, kai jos auga ne laisvai pavieniui plaukiodamos, bet prisitvirtinusios viena prie kitos arba įvairių gyvų ar negyvų paviršių. Tai leidžia apsisaugoti nuo nepalankių aplinkos veiksnių, tokių kaip imuninės sistemos atakos ar antibiotikai. Dabar jau žinoma, kad bioplėvelių formavimas būdingas didžiajai daugumui

bakterijų, ne išimtis ir *K. pneumoniae,* kuri ypač mėgsta suformuoti bioplėveles ant invazinių medicininių prietaisų, pavyzdžiui, kateterių ar endotrachėjinių vamzdelių. Tačiau bioplėvelių formavimas yra itin sudėtingas procesas, ir daug veiksnių *K. pneumoniae* nebuvo žinoma prieš pradedant šį darbą, todėl tai buvo pasirinkta kaip viena pagrindinių tyrimų krypčių.

Pirmoje darbo dalyje pristatau naują metodą FlexiPeg, skirtą bakterijų bioplėvelėms, kurios suformuojamos ant įvairių negyvų paviršių, tirti. Mūsų sukurtas įrenginys pagaminamas 3D spausdinimo technologijos dėka, todėl galimas įvairus skirtingų medžiagų ir paviršių pasirinkimas, priklausomai nuo eksperimento tikslo. FlexiPeg įrenginys yra nebrangus, daugkartinio naudojimo ir leidžia žymiai lengviau auginti didelį kiekį nepriklausomų bioplėvelių, taip pat pasirinkti skirtingas medžiagas, pavyzdžiui, silikoną, kuris dažnai naudojamas kateterių gamyboje.

Antroje darbo dalyje tyrinėjome, kaip vienas *K. pneumoniae* klonas keitėsi ”šokinėdamas” nuo vieno paciento prie kito Upsalos universitetinės ligoninės protrūkio 2005-2010 metu. Iš viso ištyrėme 110 šio klono variantų viso genomo sekoskaitos būdu ir įvairiais eksperimentiniais metodais, kad nustatytume, kaip keitėsi šio klono patogeniškumas, gebėjimas formuoti bioplėveles ir tolesnis atsparumo antibiotikams vystymasis. Genetinė analizė parodė, kad klonui buvo būdinga individuali evoliucija, reiškianti, kad dauguma mutacijų buvo atrinktos greičiausiai dėl prisitaikymo kiekviename organizme.

Trečioje darbo dalyje panaudojome eksperimentinės evoliucijos metodą, kad atrinktume mutacijas, lemiančias geresnį bioplėvelių formavimą laboratorinėje aplinkoje. Eksperimentinė evoliucija leidžia pamatyti, kaip natūralioji atranka veikia realiu laiku. Trys skirtingos *K. pneumoniae* bakterijų padermės sugebėjo labai greitai išvystyti itin gerą bioplėvelių formavimą, nepriklausomai įgydamos panašias genetines mutacijas, veikiančias įvairias ląstelės paviršiaus struktūras ar kitus svarbius procesus. Itin svarbu tai, kad šios mutacijos persipina su tomis, kurias nustatėme realaus ligoninės protrūkio metu, todėl leidžia manyti, kad jos natūraliosios atrankos buvo atrinktos būtent dėl poveikio bioplėvelių formavimui.

Ketvirtojoje darbo dalyje detaliau tyrinėjome vieną iš naujo, anksčiau neaprašyto toksiško baltymo mutacijų, kurią atradau trečiojoje darbo dalyje. Molekuliniais metodais parodėme, kad ši specifinė mutacija pakeičia baltymo toksiškumą ir stabilumą. Dėl šių pokyčių kinta toksino ir jį neutralizuojančio antitoksino kiekis ir santykis ląstelėje, ir vietoje ląstelės nužudymo „įjungiama“ bioplėvelių programa: pavyzdžiui, gaminama daugiau fimbrijų, kuriomis prisikabinama prie įvairių paviršių, taip pat išskiriama daug užląstelinių polisacharidų.

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