Investigation of One Health-related high-risk clonal lineages of multidrug-resistant *Escherichia coli* and carbapenemase-producing *Klebsiella pneumoniae* reveals key factors for their success

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"*You cannot stop the waves, but you can learn to surf*"
Jon Kabat-Zinn

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ABSTRACT

Antimicrobial resistance (AMR) is of paramount importance in the context of One Health, an integrated and unifying approach that aims to achieve a sustainable balance in the well-being of people, domestic and wild animals, plants, and their shared environments. Whenever bacteria become resistant to the therapeutic effects of antibiotics, they can cause infections that are difficult to treat effectively, increasing the risk of severe disease progression and death. Although AMR can develop naturally over time and is *per se* "ancient", the excessive use of antibiotics in human and veterinary medicine over the past century has significantly accelerated its emergence and spread. Opportunistic Gram-negative enterobacteria, particularly *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) strains, increasingly exhibit resistance to multiple classes of clinically used antibiotics, thus presenting multidrug-resistant (MDR) phenotypes. To make matters worse, some of these strains combine multidrug resistance with high-level virulence, posing a threat to both immunocompromised and healthy individuals. Consequently, MDR *E. coli* and *K. pneumoniae* have been designated as high-risk pathogens by the World Health Organization, underscoring the urgent need for new antibiotic development.

This thesis is motivated by the fact that only a limited number of international high-risk clonal *E. coli* and *K. pneumoniae* lineages stand out across all One Health dimensions and dominate the broad pool of MDR enterobacteria. While we only know little about the underlying drivers and contributing factors impacting their occurrence, emergence, and adaptation across different ecologies, this thesis employs a diverse range of bioinformatics and phenotypic approaches to identify the key factors important for the success of these lineages, also in rather under-explored settings. It includes three main components: (i) the analysis of genomic survey data of MDR *E. coli* isolates from ecologies in sub-Saharan Africa, (ii) the application of functional genomics and phenotyping techniques to characterize bacterial virulence and assess its clinical relevance in a food-borne *E. coli* strain, and (iii) the investigation of evolutionary pathways that promote the development of resistance to a novel drug combination and exploring compensatory mechanisms in a *K. pneumoniae* strain. To achieve these objectives, this research integrates genomics and transcriptomics with molecular biology and functional studies encompassing a comprehensive set of *in vitro* and *in vivo* virulence and resilience assays to explore MDR bacteria in-depth.

We provide compelling evidence for the broad occurrence of successful high-risk clonal lineages in the One Health context and their circulation among clinics, wildlife, and food in international locations. In the first study, we isolated extended-spectrum β-lactamase (ESBL)-producing *E. coli* strains from houseflies collected from various wards at the University Teaching Hospital of Butare (Rwanda). In a follow-up study, we then examined in-depth the genomes of additional ESBL-producing *E. coli* from the same clinic and obtained from hospitalized patients, their caregivers, associated community members, and pets. The analyses revealed that the sample sets from this sub-Saharan African context consisted predominantly of globally recognized *E. coli* lineages, including sequence types (ST)131, ST167, ST410, and ST617. They play a pivotal role in the further dissemination and stabilization of AMR across diverse habitats within the One Health context. Moreover, our genomic results emphasize that these One Health-related high-risk clonal lineages exhibit the ability to successfully combine multidrug resistance with high-level bacterial virulence.

To gain a more detailed understanding of the sophisticated interplay of virulence and AMR, we developed and refined a set of *in vitro* and *in vivo* methods for virulence phenotyping. These methodologies enabled us to characterize pathogens based on crucial clinical aspects such as biofilm formation, siderophore secretion, resistance to complement-mediated killing, and their capacity to cause mortality in *Galleria mellonella* larvae. By using a food-borne *E. coli* strain from an internationally recognized high-risk clonal lineage, we verified the remarkable combination of a MDR phenotype with clinically significant virulence properties, including synthesis of curli fibers and cellulose as part of biofilm formation, extensive secretion of siderophores, resilience against complement-containing human serum and pronounced mortality in the infection model.

Nevertheless, the success of One Health-related high-risk clonal lineages does not rely solely on an “ideal” synergistic interplay between bacterial virulence and AMR. It also depends on their ability to rapidly mitigate the fitness costs associated with AMR acquisition, as these costs manifest in the form of reduced competitiveness and virulence in the absence of antibiotics. However, this is at odds with the observation of the global distribution of One Health-related high-risk clonal lineages across various One Health dimensions, even in environments with expectedly low selection pressures. To comprehensively address this, we conducted experimental evolution studies selecting for ceftazidime-avibactam-resistant mutants, which illuminated the rapid adaptations to changing environments. The adaptations and compensatory mechanisms were seemingly driven by major bacterial regulators, including the envelope stress response regulator RpoE on genomic and transcriptomic levels.

In conclusion, the results of this thesis shed light on the fundamental principles that govern the character and interplay between AMR and bacterial virulence and advance our understanding of the contributors and drivers of successful MDR international high-risk clonal lineages in the One Health context. This is also important for effective and alternative intervention strategies to prospectively further address the global threat of AMR.

ZUSAMMENFASSUNG

Die Problematik der antimikrobiellen Resistenz (AMR) steht im Mittelpunkt des One-Health-Konzepts. Dieses Konzept umfasst einen ganzheitlichen Ansatz zur Sicherstellung eines nachhaltigen Gleichgewichts für das Wohlergehen von Menschen, domestizierten und wildlebenden Tieren, Pflanzen sowie ihrer gemeinsamen Umwelt. Wenn Bakterien gegen die therapeutische Wirkung von Antibiotika resistent werden, können sie Infektionen verursachen, die nur schwer wirksam zu behandeln sind und das Risiko eines schwerwiegenden Krankheitsverlaufs oder sogar des Todes erhöhen. Obwohl sich AMR im Laufe der Zeit natürlich entwickeln kann und daher an sich „vorzeitlich" ist, hat der übermäßige Einsatz von Antibiotika in der Human- und Veterinärmedizin im letzten Jahrhundert ihr Auftreten und ihre Verbreitung erheblich beschleunigt. Opportunistische gramnegative Enterobakterien, darunter insbesondere Stämme von *Escherichia coli* (*E. coli*) und *Klebsiella pneumoniae* (*K. pneumoniae*), weisen zunehmend Resistenzen gegen mehrere Klassen klinisch eingesetzter Antibiotika auf und zeigen somit multiresistente (MR) Phänotypen. Das Problem wird dadurch verschärft, dass einige dieser Stämme nicht nur MR, sondern auch hochvirulent sind, was sowohl für immungeschwächte als auch für gesunde Individuen ein ernsthaftes Risiko darstellt. Aus diesem Grund wurden MR *E. coli* und *K. pneumoniae* von der Weltgesundheitsorganisation als Hochrisikoerreger eingestuft, was den dringenden Bedarf an der Entwicklung neuer Antibiotika unterstreicht.

Diese Dissertation ist durch die Tatsache motiviert, dass nur eine begrenzte Anzahl internationaler Hochrisiko-Klonlinien von *E. coli* und *K. pneumoniae* in allen Dimensionen des One-Health-Konzepts prävalent sind und die große Gruppe der MR Enterobakterien dominieren. Obwohl nur wenig über die zugrundeliegenden bakteriellen Triebkräfte und Faktoren bekannt ist, die ihr Auftreten, ihre Evolution und ihre Anpassung in verschiedenen Ökosystemen beeinflussen, verwendet diese Dissertation eine Vielzahl bioinformatischer und phänotypischer Methoden, um die Schlüsselfaktoren zu identifizieren, die für den Erfolg dieser klonalen Linien, auch in weniger erforschten Umgebungen, entscheidend sind. Die Arbeit gliedert sich daher in drei Hauptkomponenten: (i) die Analyse genomischer Daten von MR *E. coli* Stämmen aus Ökosystemen in Afrika südlich der Sahara, (ii) die Anwendung funktioneller Genomik und Phänotypisierung zur Charakterisierung der bakteriellen Virulenz und zur Bewertung der klinischen Relevanz am Beispiel eines *E. coli* Stammes aus rohem Fleisch und (iii) die Untersuchung der evolutionären Wege, die die Resistenzentwicklung gegenüber einer neuartigen Kombination von Wirkstoffen begünstigen, einschließlich der Untersuchung kompensatorischer Mechanismen in einem *K. pneumoniae* Stamm. Um diese Ziele zu erreichen, werden Genomik und Transkriptomik mit Molekularbiologie und funktionellen Studien kombiniert, einschließlich einer umfassenden Reihe von *in vitro*- und *in vivo*-Virulenz- und Resilienztests, um MR Bakterien im Detail zu untersuchen.

Unsere Forschung präsentiert überzeugende Belege für das breitete Vorkommen von erfolgreichen Hochrisiko-Klonlinien im One-Health-Kontext, die sich in Krankenhäusern, der Tierwelt und Lebensmitteln an verschiedenen internationalen Standorten verbreiten. In unserer ersten Studie isolierten wir *extended-spectrum β-lactamase* (ESBL)-produzierende *E. coli* Stämme aus Stubenfliegen, die in verschiedenen Abteilungen des Universitätskrankenhauses in Butare (Ruanda) gesammelt wurden. In einer anschließenden Studie analysierten wir die Genome weiterer ESBL-produzierender *E. coli* Stämme aus derselben Klinik. Diese Stämme wurden sowohl von

hospitalisierten Patienten, deren Betreuern sowie von Familienmitgliedern und Haustieren isoliert. Die Analysen zeigten, dass sich die Proben aus diesem subsaharisch-afrikanischen Kontext hauptsächlich aus international anerkannten *E. coli* Klonlinien zusammensetzten, einschließlich der Sequenztypen (ST)131, ST167, ST410 und ST617. Diese Klonlinien spielen daher eine entscheidende Rolle bei der weiteren Verbreitung und Konsolidierung von AMR in verschiedenen Habitaten des One-Health-Kontextes. Darüber hinaus unterstreichen unsere genomischen Ergebnisse, dass diese One-Health-bezogenen Hochrisiko-Klonlinien die Fähigkeit besitzen, erfolgreich Multiresistenz mit hoher bakterieller Virulenz zu kombinieren.

Um dieses komplexe Zusammenspiel von Virulenz und AMR besser zu verstehen, haben wir eine Reihe von *in vitro*- und *in vivo*-Methoden zur Phänotypisierung der Virulenz entwickelt und optimiert. Diese Methoden ermöglichen die Charakterisierung von MR Bakterien hinsichtlich klinisch relevanter Aspekte, wie der Biofilmbildung, der Sekretion von Siderophoren, der Resistenz gegen Komplement-vermittelte Abtötung und ihrer Fähigkeit, Infektionen in *Galleria mellonella* Larven hervorzurufen. Unter Verwendung eines *E. coli* Stammes, der zu einer international anerkannten Hochrisiko-Klonlinie gehört und in Lebensmitteln gefunden wurde, konnten wir die bemerkenswerte Kombination eines MR Phänotyps mit klinisch bedeutsamen Virulenzeigenschaften, einschließlich der Synthese von Curli-Fasern und Zellulose als Teil der Biofilmbildung, einer ausgeprägten Siderophor-Sekretion, der Widerstandsfähigkeit gegenüber menschlichem Serum und einer deutlichen Mortalität im Infektionsmodell bestätigt.

Der Erfolg von One-Health-bezogenen Hochrisiko-Klonlinien beruht jedoch nicht nur auf einem „idealen“ synergistischen Zusammenspiel zwischen bakterieller Virulenz und AMR. Vielmehr resultiert er auch aus der Fähigkeit der MR Bakterien, die mit dem Erwerb von AMR verbundenen Fitnesskosten schnell zu reduzieren, um ihre Konkurrenzfähigkeit und Virulenz in Abwesenheit von Antibiotika aufrechtzuerhalten. Um dies umfassend zu untersuchen, haben wir experimentelle Evolutionsstudien durchgeführt, bei denen Mutanten selektiert wurden, die gegen Ceftazidim-Avibactam resistent sind. Diese Mutanten zeigten schnelle Anpassungen an veränderte Umweltbedingungen. Die Anpassungen und Kompensationsmechanismen scheinen auf genomischer und transkriptomischer Ebene durch wichtige bakterielle Regulatoren, wie den Regulator der membranständigen Stressantwort RpoE, gesteuert zu werden.

Zusammenfassend tragen die Ergebnisse dieser Dissertation dazu bei, grundlegende Prinzipien aufzuklären, die den Charakter und die Interaktion von AMR und bakterieller Virulenz bestimmen. Gleichzeitig erweitern sie unser Verständnis der Faktoren und treibenden Kräfte erfolgreicher internationaler MR Hochrisiko-Klonlinien im One-Health-Kontext. Diese Erkenntnisse sind auch wichtig für die Entwicklung effektiver und alternativer Interventionsstrategien, um der globalen Bedrohung durch AMR proaktiv zu begegnen.

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LIST OF ABBREVIATIONS

AMR ..... Antimicrobial resistance
CAZ-AVI ..... Ceftazidime-avibactam
cKp ..... "Classical" *Klebsiella pneumoniae*
CPS ..... Capsule polysaccharide
E. coli ..... *Escherichia coli*
EE ..... Experimental evolution
EPS ..... Extracellular polymeric substance
ESBL ..... Extended-spectrum β-lactamase
EU ..... European Union
ExPEC ..... Extraintestinal pathogenic *Escherichia coli*
FAO ..... Food and Agriculture Organization of the United Nations
G. mellonella ..... *Galleria mellonella*
HGT ..... Horizontal gene transfer
hvKp ..... Hypervirulent *Klebsiella pneumoniae*
Inc ..... Incompatibility group
InPEC ..... Intestinal pathogenic *Escherichia coli*
K type ..... Capsule type
K. pneumoniae ..... *Klebsiella pneumoniae*
KPC ..... *Klebsiella pneumoniae* carbapenemase
LMICs ..... Low- and middle-income countries
LPS ..... Lipopoylsaccharide
MBL ..... Metallo-β-lactamase
MDR ..... Multidrug-resistant
NSABB ..... National Science Advisory Board for Biosecurity
ori ..... Origin of replication
P. aeruginosa ..... *Pseudomonas aeruginosa*
PBP ..... Penicillin-binding protein
PDR ..... Pan-drug-resistant
RNAP ..... Bacterial core RNA polymerase complex
S. enterica ..... *Salmonella enterica*
S. typhimurium ..... *Salmonella typhimurium*
sRNA ..... Small non-coding RNA
ST ..... Sequence type
UNEP ..... United Nations Environment Programme
USA ..... United States of America
VF ..... Virulence factor
WGS ..... Whole-genome sequencing
WHO ..... World Health Organization
WOAH ..... World Organization for Animal Health
XDR ..... Extensively drug-resistant

PREFACE

In 2023, we celebrate the 95th anniversary of the discovery of penicillin by the Scottish physician and microbiologist Sir Alexander Fleming (1881–1955) [1], a discovery that revolutionized medicine and ushered in the modern era of β-lactam antibiotics. The highly effective therapy of bacterial infections made possible by penicillin led many to believe that infectious diseases would become a thing of the past. It was believed that they would eventually be eradicated from the entire human population [2]. However, this hope was dashed by the rapid emergence of resistant bacteria that no longer responded to penicillin, even when treated with higher doses [3]. In response, academia and industry worked hard to discover, develop, and deploy novel classes of antibiotics. As a result, new antibiotic drugs entered the market and literally flooded hospitals, veterinary clinics, and non-clinical settings.

Genes conferring antimicrobial resistance (AMR) can be traced back millions of years [4–6] and probably originate from *Actinobacteria* [7]. However, the use of antibiotics by humans has exerted selection pressures on the microbiosphere that favor the emergence and spread of antimicrobial-resistant bacteria [8]. Multidrug-resistant (MDR) proteobacterial representatives such as *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) from the order *Enterobacterales* [9], which are resistant to at least three clinically used antibiotics [10], become increasingly common [11–13]. These pathogens may cause recalcitrant and difficult-to-treat infections that often lead to prolonged hospital stays, high patient mortality [14, 15], and significant economic consequences [16–18]. In 2019 alone, antimicrobial-resistant bacteria were responsible for the deaths of 3.62–6.57 million people worldwide [19], and mortality rates from infections caused by antimicrobial-resistant bacteria are predicted to exceed those from cancer and cardiovascular disease by 2050 [20, 21]. Despite the growing threat, the number of pharmaceutical companies investing in antibiotic development has decreased significantly since the “golden age” of antibiotic research (approximately 1940–1962) [22]. In other words, the antibiotic drug pipeline is drying up, which could impede further innovation [23–26]. To refocus research and development related to new antibiotics, the World Health Organization (WHO) has designated several MDR bacteria, including those belonging to *E. coli* and *K. pneumoniae*, as critical priority pathogens for which effective and reliable therapeutic strategies are urgently needed [26, 27].

The lack of available therapeutic options and the spread of AMR pose a significant threat to public health. Yet evidence suggests that global antibiotic consumption will remain high [28] and double by 2030, particularly in low- and middle-income countries (LMICs) in Africa, Asia, and Latin America (all grouped together as the Global South) [29, 30]. However, many of these antibiotics are used in commercial animal husbandry rather than in the treatment of human patients [31]. As a result, millions of tons of antibiotics are released annually, exposing humans, animals, and ecosystems to antibiotics, their residues, and active metabolites [32]. It is inevitable that MDR bacteria are widespread not only in humans, but also in pets, livestock, wildlife, water, soil, and food (e.g., [33–50]). Consequently, the global spread of AMR may herald an era that will go down in human history as the post-antibiotic era [51]. Thus, the central question is no longer whether to tackle AMR, but rather what are the drivers of its widespread spread and how to address them.

1 INTRODUCTION

1.1 Antimicrobial resistance as a quintessential One Health issue

The term “evolution” refers to the process by which inherited characteristics of biological populations change over successive generations. In a narrow sense, evolution is the result of natural selection acting on a bacterial population in response to selection pressures such as exposure to antimicrobials, competition for resources, and environmental changes [52]. Since the discovery of antibiotics, their overuse and misuse have accelerated the emergence of antimicrobial-resistant bacteria [53], highlighting the importance of antibiotic efficacy as a limited resource, similar to other limited resources such as energy, minerals, and clean potable water [54, 55].

The increasing prevalence of AMR and the resulting limitations in therapeutic options require a coordinated, interdisciplinary, collaborative, and multisectoral approach. The One Health approach, which recognizes the interconnections between human, animal, and environmental health, has gained international recognition as an innovative and practical way to combat AMR [56–59]. Although the concept of One Health is relatively new, its origins can be traced back to the late 1800s when Rudolf Virchow (1821–1902), a German physician and pathologist, first proposed the link between human and animal health after discovering that human and animal disease processes were interrelated [60]. Virchow coined the term “zoonosis” to describe infectious diseases transmitted between vertebrate animals and humans [61].

The need for collaboration among different sectors to effectively address health problems was recognized by public health advocates as early as the 1950s, and many countries have established programs and agencies to promote collaboration between human and animal health. Today, One Health represents a paradigm shift from disease-centered medicine to a comprehensive view of health that encompasses multiple aspects of human, animal, and environmental health, including gentrification, rural development, health economics, chronic noncommunicable diseases, food safety, potentially pandemic (zoonotic) contagious diseases, and AMR [62].

Although the use of antibiotics is imperative for the treatment of bacterial infections in humans and animals, it poses a threat to health because antibiotic use appears to correlate with the spread of AMR. Wherever antibiotics are used, there are often large reservoirs of resistant bacteria and their associated resistance genes. These reservoirs include humans and their local environments (both in hospitals and non-clinical settings [63, 64]), as well as animals, farms, aquaculture, water, soil, and many other ecological niches [65]. However, evidence suggests that AMR genes are present not solely due to contemporary human activities, as they have existed prior to the commercial era of antibiotics [4–6]. Instead, anthropogenic perturbations drive the selection of these genes and favor the transmission and spread of AMR [63, 66–71]. These disturbances have blurred the boundaries among different habitats and led to the homogenization of biospheres adapted to human needs, facilitating the exchange and transmission of MDR bacteria to wildlife and *vice versa* [72–76].

AMR is a naturally occurring process that is influenced by selection pressures in the microbiosphere (e.g., when antibiotics are taken too frequently, for too short or long a period, or improperly). As a result, the use of antibiotics in both humans and animals poses a significant health threat and should always

be critically scrutinized, especially considering that humans and animals share the same antibiotic drug pool [77]. However, antibiotics used in veterinary medicine differ from those used in human medicine in terms of dosage, frequency of administration, and intended use [78]. In human medicine, antibiotics are primarily applied to treat clinical infections in individual patients, with limited prophylactic use in groups (e.g., to prevent meningococcal disease [79]) or in individuals (e.g., perioperatively) [80]. Nevertheless, antibiotics are often overused, inappropriately prescribed for viral infections that cannot be treated with antibiotics [81], or sold over-the-counter without a prescription [82, 83]. This holds significant potential for reducing antibiotic use and healthcare costs in human medicine [84, 85].

In commercial animal husbandry, bacterial outbreaks can have devastating economic consequences, leading to the administration of broad-spectrum antibiotics to entire herds to prevent disease in individual animals before they become infected. In addition to therapeutic, prophylactic, and even metaphylactic use, in which antibiotics are administered to a group of animals at risk of becoming infected due to exposure to a pathogen even when most animals show no signs of infection, subtherapeutic doses of antibiotics are also used for growth promotion or fattening of animals [86, 87], a practice that has even been used in humans until the mid-twentieth century [88]. The seemingly inescapable dilemma is that the excessive and incorrect use of antibiotics in one medical discipline impacts the other. For example, it is estimated that approximately 1,500 human deaths caused by MDR bacteria are attributable to the administration of third-generation cephalosporins in poultry each year in Europe [89]. The European Union (EU) recognized this trend and tightened veterinary antibiotic regulations in 2006 by banning prophylaxis and growth promotion in commercial animal husbandry [90]. However, many of the other countries from which the EU imports food and live animals do not follow this positive example [30]. Consequently, livestock and related products continue to serve as important reservoirs of harmful MDR bacteria [65, 91, 92].

Resistance to antibiotics is a global problem that poses significant challenges to the treatment of infectious diseases and results in prolonged hospital stays, higher medical costs, and increased mortality. This has led many to refer to AMR as a pandemic [93]. However, unlike a pandemic that affects a large proportion of the global population (e.g., the SARS-CoV-2 pandemic), the impact of AMR is highly variable and depends on a number of factors, including the pathogen involved, the antimicrobial drugs used, and the capacity of the local healthcare system [14, 18, 19]. Therefore, One Health recognizes that AMR does not respect traditional boundaries between human and animal health or between human health and the environment. As a result, it seeks to address this health issue at the intersection of these three domains and advocates for an integrated approach that involves collaboration between different sectors and disciplines at the local, regional, and global levels [59]. However, the history of efforts to address AMR across the One Health dimensions shows remarkable variation among countries in the speed and efficiency with which they implement the One Health approach [94–96]. In March 2022, the WHO, the World Organization for Animal Health (WOAH), the Food and Agriculture Organization of the United Nations (FAO), and the United Nations Environment Programme (UNEP) reaffirmed the importance of One Health and formed an alliance to promote harmonization of One Health-related interventions. Nevertheless, the prevalence of AMR varies widely from region to region, and some parts of the world, such as sub-Saharan Africa, have received scant attention in terms of surveillance and data availability [97, 98]. As a result, there is a lack of data on the prevalence and

extent of AMR based on surveillance data in this part of the world, although surveys suggest that AMR is a growing problem in sub-Saharan Africa and that it may be more prevalent in this region than in other parts of the world [80, 99–102].

1.2 High-risk *Escherichia coli* and *Klebsiella pneumoniae* clonal lineages drive the spread of antimicrobial resistance in the One Health context

A niche is a multidimensional and dynamic space where microorganisms such as bacteria, fungi, archaea, and protozoa colonize and interact with each other to eventually form a microbiota [103]. Darwinian competition among microorganisms has led to various strategies to gain competitive advantage, including the secretion of toxic substances such as antimicrobial compounds. In addition, naturally fermented antibiotics produced by bacteria and fungi serve as both natural weapons for gaining a competitive advantage and as means of cell-to-cell signal transduction [104, 105]. These two activities are not mutually exclusive, as they influence the interactions that determine the lifestyle and homeostasis of bacterial populations and their communities [105–109].

The widespread use of antibiotics in human and veterinary medicine has disrupted these balanced bacterial communities [110], which has led to two major evolutionary consequences [111]: (i) an evolutionary mismatch between slowly and rapidly evolving organisms, affecting the diversity of microbial populations and leading to increased abundance of MDR proteobacterial pathogens such as *E. coli* and *K. pneumoniae* [55, 110, 112–117]; and (ii) the epidemiological success of certain high-risk clonal lineages that combine high bacterial virulence and AMR [118–120].

To escape the deleterious effects of antibiotics, bacteria have evolved a multitude of defense mechanisms, such as preventing the import of drugs or increasing their export, altering the antimicrobial target itself, or producing enzymes that either destroy or inactivate the antibiotic [121]. AMR genes and mechanisms are widely distributed, but not all are present in proteobacterial pathogens [26, 122]. Resistance developed to β-lactams is a prime example of how a bacterial defense mechanism has impacted human and animal health.

Clinically, β-lactams are among the most commonly used antimicrobial agents for the treatment of infections caused by *E. coli*, *K. pneumoniae*, and other members of *Enterobacterales* in humans and animals [29, 123–125]. These drugs inhibit cell wall synthesis by covalently binding to penicillin-binding protein (PBP), resulting in lysis of exclusively bacterial cells. However, mutations in various bacterial transpeptidases resulted in functional enzymes that can hydrolyze the β-lactam ring, rendering these drugs inactive [126]. To date, 8,154 of these enzymes have been identified and grouped as β-lactamases (reported in the β-lactamase database [127], retrieved on November 22, 2023). These enzymes differ in their ability to degrade different types of β-lactam antibiotics. Some β-lactamases inactivate only certain types of β-lactams (e.g., penicillinases), while others, known as extended-spectrum β-lactamases (ESBLs), have a broader spectrum and can counteract many different types of these drugs. Among proteobacterial pathogens, the increasing prevalence of ESBLs is of particular concern [12].

Traditionally, β-lactamases are classified into four groups based on protein homology, known as Ambler classes A to D [128]. ESBLs belong almost exclusively to Ambler class A and can be defined as enzymes

that confer resistance to penicillins, first- to third-generation cephalosporins, and monobactams. Since the early 2000s, CTX-M enzymes have become extraordinarily common [35, 129, 130]. The dramatic increase in ESBL-producing *E. coli* and *K. pneumoniae* has led to a shift in prescriptions from third-generation cephalosporins and monobactams to carbapenems such as imipenem and meropenem [131]. Because of their exceptionally broad spectrum of antibiotic activity and stability to former β-lactamases, carbapenems were considered the most reliable last-resort treatment option for infections caused by ESBL-producing *Enterobacterales* in humans. Inevitably, carbapenem-hydrolyzing β-lactamases are on the rise [132–134] and resistance is conferred by carbapenemases belonging to Ambler class A (e.g., *K. pneumoniae* carbapenemase [KPC]), B (metallo-β-lactamases [MBLs] such as NDM-1), and D (e.g., OXA-48-type oxacillinases). However, evidence suggests that ESBL and carbapenemase genes are concentrated primarily in a limited number of high-risk clonal lineages of *E. coli* and *K. pneumoniae* that are distributed worldwide [135].

In the field of bacteriology, there is ambiguity in defining the terms “lineage”, “clonal lineage”, and “clone”. According to Dijkshoorn *et al*. (2000), a bacterial clone refers to a group of genetically identical bacteria descended from a single ancestor through asexual reproduction. Clones share a similar genetic makeup because bacteria reproduce by dividing into two daughter cells that are genetically identical to their parent cells (a process called binary fission). However, clones within the same clonal lineage may undergo mutations, which cause them to develop different characteristics but still belong to the same group because they have all descended from a common ancestor [136]. Accordingly, the term “international high-risk clonal lineage” refers to bacteria that have been independently isolated from a variety of hosts found worldwide and possibly at different times. In the One Health context, a clonal lineage can therefore be classified as “high-risk” if it meets the following criteria: (i) newly recognized or already established but with expansion in terms of spatial distribution and host or vector range; (ii) carrier of multiple AMR determinants; (iii) high probability of transmission following exposure to an infected host or contaminated source; (iv) long-term persistence in colonized hosts; (v) occurrence in multiple animal species, human populations, and ecologically conserved sources; and (vi) ability to cause severe disease in animals and humans [137]. This definition suggests that One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae* have successfully adapted to the human-animal-ecosystem interface, i.e., they have an enhanced ability to colonize, spread, and persist in a variety of niches [110, 138]. By way of introduction, the following two subsections briefly outline a selection of One Health-related high-risk *E. coli* and *K. pneumoniae* lineages. However, it should be noted that our knowledge to date is limited to surveys and systematic analyses of mainly well-studied areas in Australia, Europe, and the United States of America (USA) [139–143]. It is by no means clear that the same high-risk clonal lineages are prevalent in the Global South [140].

1.2.1 One Health-related high-risk *Escherichia coli* lineages

E. coli is a species of Gram-negative, facultative anaerobic, rod-shaped bacteria that occurs as commensals in the intestines of vertebrates [144, 145], but can also be found as fecal contaminants in environmental sources such as water through fecal contamination [146]. The ubiquitous occurrence of *E. coli*, its high metabolic flexibility, efficient translational machinery, and ease of handling have made it one of the most studied bacterial organisms, widely used in research and biotechnology [147–150].

However, genetic plasticity has led to considerable phenotypic variation within *E. coli* species, with a spectrum of resistance ranging from largely drug-susceptible commensals to MDR pathogenic strains [13, 151–153]. In particular, the global emergence and rapid spread of the latter have made them one of the most devastating critical pathogens in public health and veterinary medicine. Moreover, *E. coli* strains are among the most common zoonotic bacteria transmitted from animals to humans and *vice versa* [154], making pathogenic MDR *E. coli* an important issue in the One Health context.

Pathogenic *E. coli* can be categorized into two distinct phenotypic groups based on their ability to cause disease. Obligate intestinal pathogenic *E. coli* (InPEC) cause severe disease in the host gastrointestinal tract, including gastroenteritis, bloody diarrhea, and hemolytic uremic syndrome [155, 156]. InPEC are subdivided into six recognized pathotypes based on characteristic toxin production and other virulence factors (VFs) [156]. It is noteworthy that these pathotypes can overlap, as seen in a hybrid InPEC clone responsible for an outbreak in Northwestern Germany in 2011 [157]. The outbreak clone carried an enteroaggregative *E. coli* plasmid and possessed the genomic characteristics of enterohemorrhagic *E. coli* [158]. In addition, it carried the ESBL gene $bla_{\text{CTX-M-15}}$ [159], which is uncommon in InPEC but often found in facultative extraintestinal pathogenic *E. coli* (ExPEC) [130].

ExPEC are usually asymptomatic colonizers of the vertebrate gut. However, they can cause various infectious diseases such as urinary tract infections, neonatal meningitis, and bloodstream infections when they spread to normally sterile areas of the body [156, 160]. Unlike InPEC, ExPEC pathotypes cannot be demarcated solely by the presence of specific VFs alone [161], as some of these features overlap among the different ExPEC pathotypes and are also found in commensal strains (e.g., the yersiniabactin-associated gene *fuyA* [162, 163]). These factors represent strategies required for adaptation, gut colonization, and competition with other microbiota without harming the host [150]. Clinically, ExPEC are therefore classified according to the clinical symptoms they cause in patients [164–167], for example, uropathogenic *E. coli* [168] and neonatal meningitic *E. coli* [169].

Microbial genomics has become more accessible with the advent of high-throughput sequencing platforms such as Illumina and PacBio [170]), which has led to a better understanding of the impact of different subpopulations of (pathogenic) *E. coli* on humans, animals, and the environment. Phylogenetic analyzes have shown that the versatile species *E. coli* is divided into eight distinct phylogenetic groups (A, B1, B2, C, D, E, F, and G), which roughly correspond to the lifestyle of the different strains [150, 171–177]. Studies have shown that phylogroups B2 and D, and occasionally C, F, and G are strongly associated with ExPEC [176, 178]. In contrast, commensals and InPEC are typically found in groups A and B1, respectively [179–181]. Apparently, this population structure and the presence of phylogroup-specific core genes reflect different evolutionary strategies [177, 182–188]. For example, strains belonging to phylogroup B2 lack the *lsr* operon [189], a quorum sensing system required for social interactions among *E. coli* and other species of the gut microbiome [190, 191]. Upon loss of function of the *lsr* operon, corresponding ExPEC strains are able to successfully invade new (intestinal) niches, promote a change in the microbial homeostasis (especially in combination with prolonged antibiotic exposure), and then become established in the microbiota [192].

Although extraintestinal infections can be caused by many different phylogenetic backgrounds (more than 200 different ExPEC sequence types [STs] have been identified [193]), most ExPEC-associated

infections in humans and animals are caused by only a few dominant clonal lineages, including ST38, ST131, ST167, ST405, ST410, ST617, ST648, and others (e.g., [140, 194–198]). Among the One Health-related high-risk *E. coli* lineages, ST131 is still the predominant pathogenic lineage in mammals [199] and represents a frequently cited example of AMR in clonal evolution [153]. As previous studies on ST131 have shown, this high-risk clonal lineage is not a uniform entity, but rather exhibits a three-clade structure determined by the possession of variants of the type 1 fimbrial adhesin gene *fimH*: H41, H22, and H30 (alternatively referred to as clades A, B, and C, respectively) [200]. The main AMR culprits are ST131 strains from the dominant clade C, which have evolved from a susceptible basal component (ST131-H30S) to a fluoroquinolone-resistant and ESBL-producing sister clade ST131-H30R [187, 200–202]. Further analysis revealed that ST131-H30R has split and is now almost completely replaced by two sister clades. By acquiring different plasmids, the resistant ST131-H30 strains differ in their ESBL gene content. Originally, ST131-H30R carried *bla*$_{\text{CTX-M-14}}$, whereas its sister clades ST131-H30R1 and ST131-H30Rx carry *bla*$_{\text{CTX-M-27}}$ and *bla*$_{\text{CTX-M-15}}$, respectively [200, 203].

Recent studies have significantly enhanced our understanding of the evolutionary success of high-risk clonal lineages of *E. coli* [150, 204]. ST410 and ST648 are prominent examples of such clonal lineages from a One Health perspective [38, 204–207]. Notably, ST410 has a similar population structure as ST131, as evidenced by differences in *fimH* alleles. The ST410-B/H24 sublineage is the most important in terms of AMR, with the fluoroquinolone-resistant variant ST410-B2/H24R arising due to mutations in *gyrA* and *parC* coinciding with the widespread use of fluoroquinolones in the 1980s. An ESBL-producing variant (ST410-B3/H24Rx) emerged with admission of *bla*$_{\text{CTX-M-15}}$, followed by the carbapenem-resistant ST410-B4/H24RxC, which encodes carbapenemases in addition to *bla*$_{\text{CTX-M-15}}$ [208, 209]. Today, ST410 clones with acquired carbapenemase genes, such as *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-181}}$, and *bla*$_{\text{NDM-5}}$, are highly prevalent [208–210].

Similar to *E. coli* ST131, MDR ST410 clones may also cause a wide spectrum of infectious diseases in humans and animals, ranging from uncomplicated urinary tract infections to respiratory failure and septic shock (e.g., [206, 211–213]). Although it is often assumed that AMR leads to a reduction in bacterial virulence [214], there is evidence that certain high-risk clonal lineages possess both numerous AMR genes and a large repertoire of virulence-associated attributes [215]. Mobile genetic elements, i.e., DNA sequences that can move within genomes and between bacterial cells, appear to play an important role in the co-carriage of AMR determinants and VFs in One Health-related high-risk clones [207, 209, 215–217]. The genetic foundations and plasmid fixation in One Health-related high-risk clonal lineages are further discussed below.

1.2.2 High-risk *Klebsiella pneumoniae* lineages in the One Health context

Carbapenemases are now widespread in *Klebsiella* spp., which are abundant in various environmental niches such as soil, water, and surfaces [218–221], and also commonly colonize the gastrointestinal tract, respiratory tract, mouth and skin of mammals [222–224]. However, the genus *Klebsiella* includes several species that can cause severe opportunistic infection. Among these species with human and animal pathogenic potential, *K. pneumoniae* is considered the greatest health threat [142, 225–229]. The “classical” *K. pneumoniae* (cKp) infections are pneumonia, and urinary tract and wound infections, each of which can lead to bacteremia [230]. Although *K. pneumoniae* is ubiquitous in nature, suggesting

that the environment is a reservoir for this harmful opportunist, evidence suggests that healthcare-associated infections are usually caused by cKp strains that have already colonized the host [231–234], whereby zoonotic transmissions may have preceded [223, 224]. Vulnerable patient groups such as neonates, the elderly, those with implanted medical devices or surgical wounds, and immunocompromised patients are most at risk. Obviously, the critical condition of these patients emphasizes the need for reliable and effective treatment. However, therapeutic options are increasingly limited due to the notorious propensity of cKp to acquire multiple AMR determinants [13, 235]. This leads to extensively drug-resistant (XDR) or eventually pan-drug-resistant (PDR) clones that are no longer treatable with available antibiotics [10], rendering infection by these strains a major challenge for clinicians.

All *K. pneumoniae* strains are archetypally virulent and possess chromosomally encoded VFs such as the siderophore enterobactin (*ent* locus), fimbrial adhesins (*fim* and *mrk* loci), and robust capsular polysaccharides (encoded by a variety of genes) [236]. In addition, data suggest that other virulence-associated genes are concentrated in specific subpopulations of *K. pneumoniae* that represent hypervirulent strains (hvKp) [237]. Unlike cKp, infections caused by hvKp are usually acquired in the community and occur in otherwise healthy individuals [238] and, interestingly, frequently in people with diabetes mellitus [239–243]. The hvKp infections typically manifest in body sites unusual for *Enterobacterales* (e.g., liver and biliary tract, lung, eye, central nervous system, and musculoskeletal system) and can spread metastatically, eventually leading to severe bloodstream infections with high mortality. Clinically, hvKp is characterized by the invasive nature of the potentially life-threatening infection and only rare resistance to antibiotics [244] (with the exception of ampicillin, to which *K. pneumoniae* is intrinsically resistant [245]). HvKp was originally discovered in the Asia-Pacific region (e.g., Taiwan [246] and South Korea [247]), but increasing numbers of infections caused by hypervirulent strains are now being reported worldwide [248–254]. However, there is no microbiological consensus on the classification of hypervirulence [255]. Traditionally, hypervirulence is defined by exuberant capsule production (hypermucoviscosity) in terms of a positive string test, in which a colony stretches more than 5 mm when pulled from a plate [256]. Due to the high variability of hypermucoviscosity, this definition is less accurate [257, 258]. Therefore, recent studies recommend the determination of genetic biomarkers such as *peg-344* (metabolite transporter), *iucA* (aerobactin), and plasmid-encoded *rmpA* and *rmpA2* (regulators of mucoid phenotype) [259, 260] in combination with *in vitro* and *in vivo* phenotyping to distinguish between cKp and hvKp [261].

Just like ExPEC, the *K. pneumoniae* population consists of epidemiologically successful clonal lineages. Clonal lineages commonly associated with XDR cKp include ST11, ST101, ST147, ST258, and ST512 [231], whereas STs commonly associated with hvKp are ST23, ST65, ST86, and ST375 [230]. One of the most dangerous One Health-related lineages is *K. pneumoniae* ST307 [262, 263]. This clonal lineage was first reported in a Pakistani clinic in 2009 [264] and has since spread throughout Europe, Asia, and the Americas [263]. Most reports have come from a variety of human clinical samples, but ST307 clones have also been detected in animals, including cats and dogs, and in environmental samples (e.g., [224, 265–270]). The first Pakistani isolates were MDR CTX-M-15-producing strains, but they remained susceptible to carbapenems. One year later, $bla_{\text{KPC-2}}$-positive and thus carbapenem-resistant ST307 clones were found in two hospitals in Texas, USA [271]. In the following

years, *K. pneumoniae* ST307 has been associated with a variety of ESBLs and carbapenemases, including *bla*$_{KPC-3}$ [272], *bla*$_{OXA-48}$ [273], *bla*$_{OXA-181}$ [274], *bla*$_{NDM-1}$ [275], and *bla*$_{VIM-1}$ [276]. It has long been a common dogma that pathogenic strains discretely belong to MDR cKp or hvKp based on their phylogenetic background [237, 277]. Increasingly, however, clonal lineages such as ST307 are spawning representatives that simultaneously exhibit both hypervirulent properties and resistance to numerous antibiotics [118, 233, 278–284]. This convergence of phenotypes appears to be due not only to the exchange of resistance and virulence plasmids [278, 280], but also to the chromosomal integration of DNA sequences of unusually great length [239, 285, 286] and the rearrangement of plasmid DNA [233, 287–291]. For example, from June 2019 to February 2020, a clonal outbreak of *K. pneumoniae* ST307 occurred in four healthcare facilities in Mecklenburg-Western Pomerania, Germany [118, 292]. The outbreak clone carried a “mosaic” plasmid encoding both AMR determinants and VFs. During the outbreak, other clonal *K. pneumoniae* lineages and *Enterobacterales* were identified carrying similar plasmid sequences [118], suggesting the deleterious role of the outbreak clone in the transmission of these plasmids within and between species and consequently the spread of AMR and virulence genes possibly across different dimensions of One Health [293].

1.3 Bacterial strategies of resistance and virulence acquisition

Exposure to antimicrobials forces many different microbial organisms to evolve survival strategies, and there are only a limited number of genes that can elicit resistance. The evolution of AMR can clearly be seen as an expression of competition among different bacterial phyla within a niche. This evolutionary process includes all Darwinian elements, such as variation (within the bacterial population), selective pressure, and inheritance to descendants. However, natural bacterial competition also contains altruistic or cooperative elements that maintain bacterial coexistence and genetic variation. Moreover, as described above, anthropogenic influence is less responsible for the general evolution of AMR [4, 6], but rather for selection and thus stabilization of AMR (i.e., the unlikelihood that AMR will decline when selection pressure is reduced [214]). In terms of stabilization, MDR One Health-related high-risk clonal lineages in particular seem to play a crucial role.

Extensive research has uncovered different evolutionary strategies of resistance acquisition, including (i) the circulation of resistance plasmids even across phylogenetic boundaries and their genetic linkage to other selection markers (e.g., heavy metals) or (ii) chromosomal mutations conferring resistance [294]. In the following two subsections, these different mechanisms are discussed in more detail using examples of MDR *E. coli* and *K. pneumoniae* lineages. It should be noted that these examples are not exhaustive and that other bacterial species may use different mechanisms to acquire resistance.

1.3.1 Genetic exchange through the circulation of plasmids

There are two distinct directions in which resistance-conferring genes can be transferred from one bacterial host to another (Figure 1). In vertical gene transfer, the material is transferred from an ancestor to descendants and therefore remains within the clone (Figure 1A). Conversely, when AMR genes are encoded on mobile genetic elements (e.g., plasmids) in a microbial community and certain species can coexist, there is an increased likelihood of horizontal gene transfer (HGT), even among different

bacterial species (Figure 1B). Indeed, antibiotic exposure can trigger the exchange of genetic material among organisms that are not necessarily in a clonal relationship [122, 295–300]. To complicate matters, other compounds that are not primarily used to treat bacterial infections (e.g., the widely used antidepressants fluoxetine and escitalopram) may also promote the spread of AMR genes by increasing HGT [301, 302].

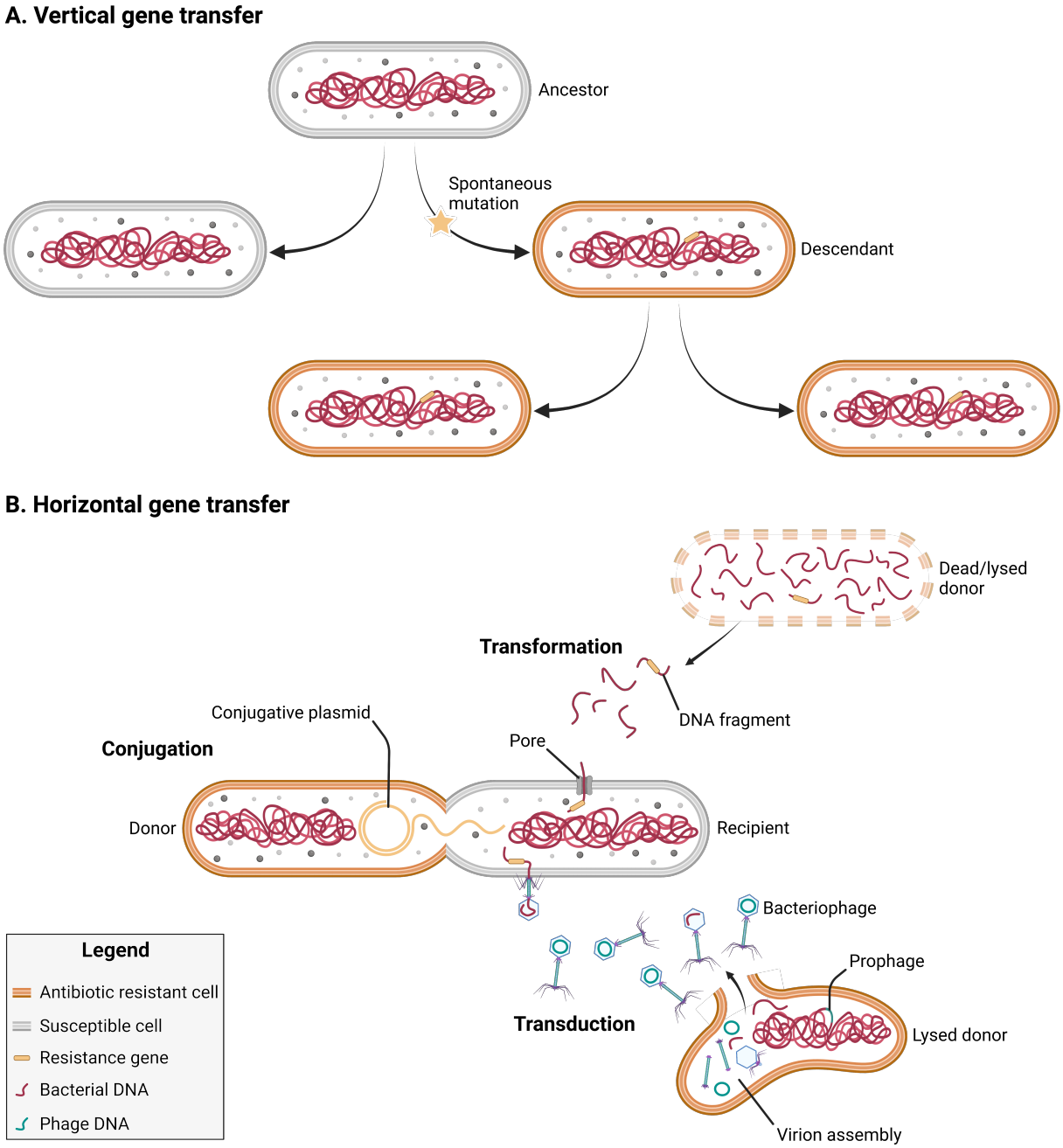

Figure 1: Development of antimicrobial resistance through gene exchange. A Vertical gene transfer is the primary mechanism of genetic inheritance in bacteria. It occurs by binary fission, in which a single bacterial cell divides into two identical daughter cells, each containing a copy of the parental genome. This process allows the accumulation of beneficial mutations, including those that confer resistance to antibiotics. **B** Horizontal gene transfer occurs when bacteria exchange genetic material, such as antimicrobial resistance determinants. The exchange of genetic material can occur by several mechanisms, including conjugation, transformation, and transduction. Conjugation involves the transfer of conjugative plasmids from a donor cell to a recipient cell, using a pilus or bridge-like structure to establish a connection and transfer genetic material between the two cells. Transformation occurs when a bacterial cell takes up free DNA, which can originate from dead or lysed bacterial cells, and integrates it into the bacterial genome through recombination. Transduction refers to the transfer of genetic material among bacterial cells by a bacteriophage vector. Such viruses can infect bacterial cells by inserting phage DNA into the bacterial chromosome as a prophage. The phages use bacterial machinery to replicate and produce new virus particles (called virion assembly), occasionally packaging host DNA. Finally, the donor cell is lysed and free phages infect a naive recipient cell, where the DNA recombines into the chromosome. Created with BioRender.com.

The three best known mechanisms of HGT in prokaryotes are transformation, transduction, and conjugation [303]. Transformation refers to the internalization of exogenous DNA and its subsequent integration into the recipient genome [304], whereas transduction involves the (random) transfer of genetic material to the recipient by a bacteriophage vector that has integrated the genetic material of a foreign host into its phage genome [305, 306]. Conjugation requires physical contact between a donor and recipient cell via a conjugation pilus through which genetic material is transferred [307].

HGT and conjugation in particular facilitate genetic diversification in *E. coli* and *K. pneumoniae*, and plasmids are one of the most important vehicles for this process [308–310]. Plasmids are extrachromosomal elements of circular DNA that replicate independently of the host genome. They drive gene flow, adaptation, and diversification within microbial populations. The plasmid replicon, the backbone of all plasmids, contains its origin of replication (*ori*), one or more replication initiation proteins, and a regulatory system using antisense RNAs or iterons to control the number of plasmids replicated by each bacterial cell. Hence, like parasites, plasmids carry genes required for self-replication, but use host machinery to accomplish their replication [311]. However, in contrast to the deleterious lifestyle of parasites, plasmids provide VFs and benefits to bacterial physiology (e.g., metabolic enzymes [312] and AMR [313]) and may also have protective properties for the host chromosome. The latter is based on the fact that transposition events tend to insert DNA at sites where insertion has previously occurred. Thus, when a plasmid is present, invading transposons preferentially integrate into the plasmid rather than the host chromosome [314]. Any insertion of genes into the bacterial chromosome may disrupt existing genomic sequences or result in large rearrangements of the chromosome [239], which may prove deleterious by altering gene expression levels or bacterial phenotype [315].

Despite the potential benefits that plasmids bring, they also impose a burden on fitness and thus reduce the reproductive rate and virulence of the host bacterium [316]. This significantly affects plasmid stability within the bacterial population. Therefore, horizontally transferred genes must provide significant benefit or they are likely to be lost quickly, not least because of the mutational propensity for deletions in bacterial genomes [317]. However, recently acquired regions often function inefficiently in their new host, so they can be energetically or physiologically costly [315], and several other factors contribute to these costs [318]. For example, there are large differences in codon usage between plasmids and the bacterial chromosome [319, 320]. The low number of certain tRNAs causes ribosomes to get stuck in foreign transcripts, which slows the elongation of nascent proteins and reduces overall cellular translation efficiency [320–322].

Contrary to expectations, some resistance plasmids (e.g., ESBL plasmids) do not necessarily reduce bacterial fitness. This seems to be particularly true for One Health-related high-risk clonal lineages [209, 216]. For example, an *E. coli* ST131-H30Rx strain showed increased fitness and virulence when carrying an ESBL plasmid with replicon type or incompatibility group (Inc)FII compared to its plasmid-free variant [215, 217, 323]. In addition, a recent study has shown that the carriage of ESBL plasmids can increase intestinal colonization in mice [324]. This appears to be due to the fact that associations between plasmid replicon types, high-risk clonal lineages, and ESBLs or carbapenemases are not random [325–327]. As indicated earlier, the evolution of *E. coli* ST131-H30Rx was driven by the acquisition of IncFII plasmids encoding CTX-M-15 [203], whereas ST410-B3/H24Rx evolved by the

acquisition of a multiple replicon type (IncFIAB-IncFII) plasmid. The emergence of ST410-B4/H24RxC was associated with the acquisition of IncX3 plasmids encoding non-MBL carbapenemases or conserved IncFII plasmids encoding MBL carbapenemases [208]. Even if these plasmids initially incurred fitness costs, adaptations between host and plasmid appear to have occurred that mitigated plasmid burden over time [328, 329]. In some cases, this plasmid-host adaptation appears to be so beneficial that certain plasmids appear to be fixed within a phylogenetic background (i.e., high stability within their clonal lineage) with very limited transmission outside the clonal complex [330]. Interestingly, the phenomenon of conjugative suppression occurs more frequently when multiple plasmids of different replicon types are in the same bacterial host than when multiple plasmids in a mixed bacterial population are in different hosts [331]. However, not all plasmids can co-exist within the same bacterial host due to their incompatibility with other plasmids that have similar replicon types. The phenomenon of plasmid incompatibility arises when multiple plasmids compete for the same replication control machinery within the host. When plasmids are compatible, their replication is regulated by separate regulatory systems that utilize distinct replication machinery. However, when plasmids share a common *ori*, the regulatory system cannot distinguish between them. This causes an artificial increase in the concentration of components of the regulatory system within the cell, leading each plasmid to falsely appear to have a higher copy number than it actually does. As a result, neither plasmid is maintained [311, 332].

Similar to *E. coli*, *K. pneumoniae* has an “open” pangenome with a small fraction of core genes (approximately 1,700 genes) and a huge pool of accessory genes (probably 100,000 protein-encoding sequences) [237]. The latter are typically acquired through extensive transmission and carriage of diverse plasmids encoding AMR determinants and VFs, respectively [277, 333, 334]. It has been reported that *K. pneumoniae* appears to be quite amenable to plasmid uptake and maintenance, resulting in larger amounts of plasmids than in *E. coli* and other *Enterobacterales* [335]. Returning to the example of the *K. pneumoniae* ST307 outbreak clone: This clone harbored five different plasmids and, interestingly, the ESBL gene $bla_{\text{CTX-M-15}}$ and the carbapenemase genes $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ were each located on different plasmids. Further analysis revealed that the largest plasmid 1 (approximately 360 kbp) had a mosaic structure, i.e., it encoded multiple resistance genes (including $bla_{\text{NDM-1}}$) and VFs, the latter commonly found on virulence plasmids of hvKp. Paradoxically, partial plasmid curing of plasmid 1 using a heat technique [336] resulted in neither a shift in growth rate nor an increase in overall fitness compared to the plasmid-carrying wild-type strain PBIO1935, despite the size of the plasmid and the metabolic burden expected as a result [118].

One may wonder what is the reason for the apparently advantageous plasmid-host combinations suggested by these examples. On the one hand, the bacterial host must develop strategies to cope with the presence of plasmids once they have successfully established themselves. In this context, transcriptional changes seem to be an effective way to reduce fitness costs, since most costs are incurred when genes encoded on plasmids are expressed [337, 338]. Recent studies have also highlighted the importance of compensatory mutations in offsetting fitness costs [339–342]. To maximize the adaptive effect of a plasmid in a given host, it is necessary to modify its gene content, adjust the level and timing of gene expression, and optimize its DNA sequence features, such as codon usage, to match those of the host [343].

On the other hand, even if one neglects the fitness costs associated with plasmids, the persistence of plasmids in bacterial communities is jeopardized by their potential loss during bacterial cell division. Moreover, plasmid genes that confer useful properties may eventually be inserted into the bacterial chromosome, while the rest is discarded [344]. However, this seems to be of minor importance considering how many accessory genes are provided by plasmids in One Health-related high-risk clonal lineages [177, 345]. To maintain their persistence even in the absence of positive selection (e.g., antibiotic treatment), plasmids use several mechanisms for post-segregation killing. These systems encode a toxin-antitoxin pair that produces a stable toxin and a less stable antitoxin molecule. In the event of plasmid loss, the antitoxin is degraded faster than the toxin, resulting in cell death. These systems can only be overcome under excessive stress conditions, ensuring high plasmid stability in the host and bacterial community [336]. In addition, plasmids can provide resistance to other selection agents such as heavy metals or biocides [45]. The continuous introduction and progressive accumulation of detergents, disinfectants, and residues from industrial pollution in the environment leads to the concomitant selection of plasmids that combine suitable resistance genes. Therefore, the spread and global dispersion (i.e., outside clinics and even in pristine environments) of AMR-associated plasmids is mediated not only by antibiotics themselves but additionally by other concurrent selection factors [346]. In addition to co-selection, resistance to heavy metals also appears to be a key factor in plasmid transmissibility, as zinc and copper ions, for example, inhibit HGT [347].

1.3.2 Chromosomal mutations conferring antimicrobial resistance

Research on the genetics of AMR has shown that pathogenic bacteria not only acquire resistance to clinically relevant doses of antibiotics through plasmids encoding appropriate resistance determinants, but can also develop resistance through chromosomal mutations. For example, there is evidence that CTX-M enzymes originally arose from chromosomally encoded cefotaximases from *Kluyvera* spp. [348, 349]. Changes in amino acid sequence (e.g., replacement of asparagine-240 with glycine observed in the progenitor of *bla*$_{\text{CTX-M-15}}$ [350]) led to an expansion of the resistance spectrum [351–354], and mobilization of these genes led to the great success of the CTX-M family within the order *Enterobacterales* [355]. This example shows that chromosomal mutations of resistance genes can lead to an expansion of the spectrum of activity. However, chromosomal mutations can also cause formerly non-resistance-associated genes to contribute to AMR. This was certainly the case with the XDR *K. pneumoniae* ST307 outbreak clone mentioned above. Phenotypic antimicrobial susceptibility testing revealed that the outbreak clone was resistant to colistin [292]. However, whole-genome sequencing (WGS) indicated that this resistance was not mediated by genetic markers such as the mobile colistin resistance (*mcr*) genes, but was due to missense mutations in transcriptional regulators of the two-component systems PhoPQ and PmrAB [118]. Usually, the cell surface of Gram-negative bacteria is strongly negatively charged so that cationic colistin is attracted. However, mutations in the two-component systems promote the attachment of positively charged 4-amino-4-deoxy-L-arabinose and phosphoethanolamine molecules to lipid A of the bacterial lipopolysaccharide (LPS). The resulting masking of the negative charge reduces the interactions of colistin with the outer bacterial membrane, leading to resistance to polymyxins [356–359]. Interestingly, comparable mutations were found in two-component systems of a colistin-resistant *E. coli* ST131-H30Rx strain derived from a hospitalized

patient [360] and an *E. coli* ST1598 strain obtained from a cloacal swab of a black-headed gull (*Chroicocephalus ridibundus*) [361], suggesting common mechanisms in resistance acquisition through chromosomal mutations.

Possession of an AMR gene is clearly beneficial to the bacterial host if the appropriate antibiotic is present. However, AMR is severely limited by the fitness costs it incurs, as these costs negatively affect the reproduction and survival of the bacterial host, thus selecting against resistance in the absence of selective pressure from the antibiotic. In addition, several studies have shown that the costs of resistance likely vary between plasmids and chromosomal mutations [316]. The latter can alter essential genes that are otherwise highly conserved, with devastating effects on bacterial fitness. In *K. pneumoniae*, for example, carbapenem resistance can result from alterations in membrane permeability due to loss of the major non-selective porin channel OmpK36 [362–365]. These mutations are known to reduce fitness and result in loss of bacterial virulence [363, 366], although mutations in the nucleotide sequences of OmpK36 are frequently detected in clinical isolates [41, 364, 367]. The wide occurrence of plasmid-encoded resistance genes and chromosomally mediated resistance within One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae*, particularly in more pristine environments with less anthropogenic perturbation, contradicts the assumption of self-limitation of AMR in the absence of antibiotics. Thus, an important question is how these high-risk clonal lineages can compensate for the deleterious costs of resistance genes.

1.3.3 Experimental evolution – A powerful tool to uncover bacterial resistance and virulence evolution

Exposure to antimicrobials may lead to bacterial AMR acquisition, which is often associated with reduced growth rates, competitiveness, and virulence [316, 318, 368, 369]. These fitness and virulence costs depend on the specific resistance mechanism and the bacterial species involved. For example, genetic mutations and phenotypic changes that confer AMR may also affect other essential bacterial proteins or processes, resulting in slower reproduction or decreased ability to metabolize nutrients [214]. However, the evolutionary process may also act as a stress-reducing mechanism driven by the potential difference between the organism's current fitness and the possibility of better fitness, i.e., returning to a steady state after a perturbation [370]. Evolutionary trajectories involve sequences of transcriptomic, epigenetic (both summarized as adaptive plasticity), and genetic adaptations that can progressively promote significant increases in fitness [371, 372]. The early stages of the evolutionary process generally rely on adaptive plasticity rather than genetic changes, such as compensatory mutations [373, 374]. In some cases, however, compensatory mutations are required to terminate and fix the process of recovery [340, 365, 375–377]. Yet evolution is not omnipotent and has its limitations [373], which arise from limited genetic variation, mutually exclusive trait combinations, or transitional states that have low fitness (i.e., fitness valleys) between the current phenotype and better phenotypes such that they cannot compete and eventually disappear [371, 378–382].

To facilitate evolutionary analysis, *in vitro* experimental evolution (EE) has become a powerful tool to study how bacterial populations evolve over time in response to specific environmental conditions or selection pressures exerted by the experimenter (Figure 2). In particular in combination with genomics, it provides snapshots of genetic diversity in evolving populations and elucidates the precise genetic

changes that drive the evolutionary process [383]. Unlike knock-out or knock-in studies, EE avoids the bias of unwanted but unavoidable mutations and instead allows to observe natural genetic variant selection under controlled conditions [384, 385]. EE encompasses a wide range of possible experimental designs in which populations are studied over multiple generations under defined and reproducible conditions [385, 386]. However, similar to natural evolution, the evolutionary change detected by EE depends on several known and unknown factors. For example, it was shown that shrinkage of the effective population size and the strength of selection pressure affect the experimental outcome due to the reduction of genetic variation and potential loss of certain genetic traits [387, 388]. In addition, resistance development and changes in bacterial fitness are highly dependent on general growth conditions as well as the duration of the experiment and the associated number of bacterial generations that can evolve [376, 389].

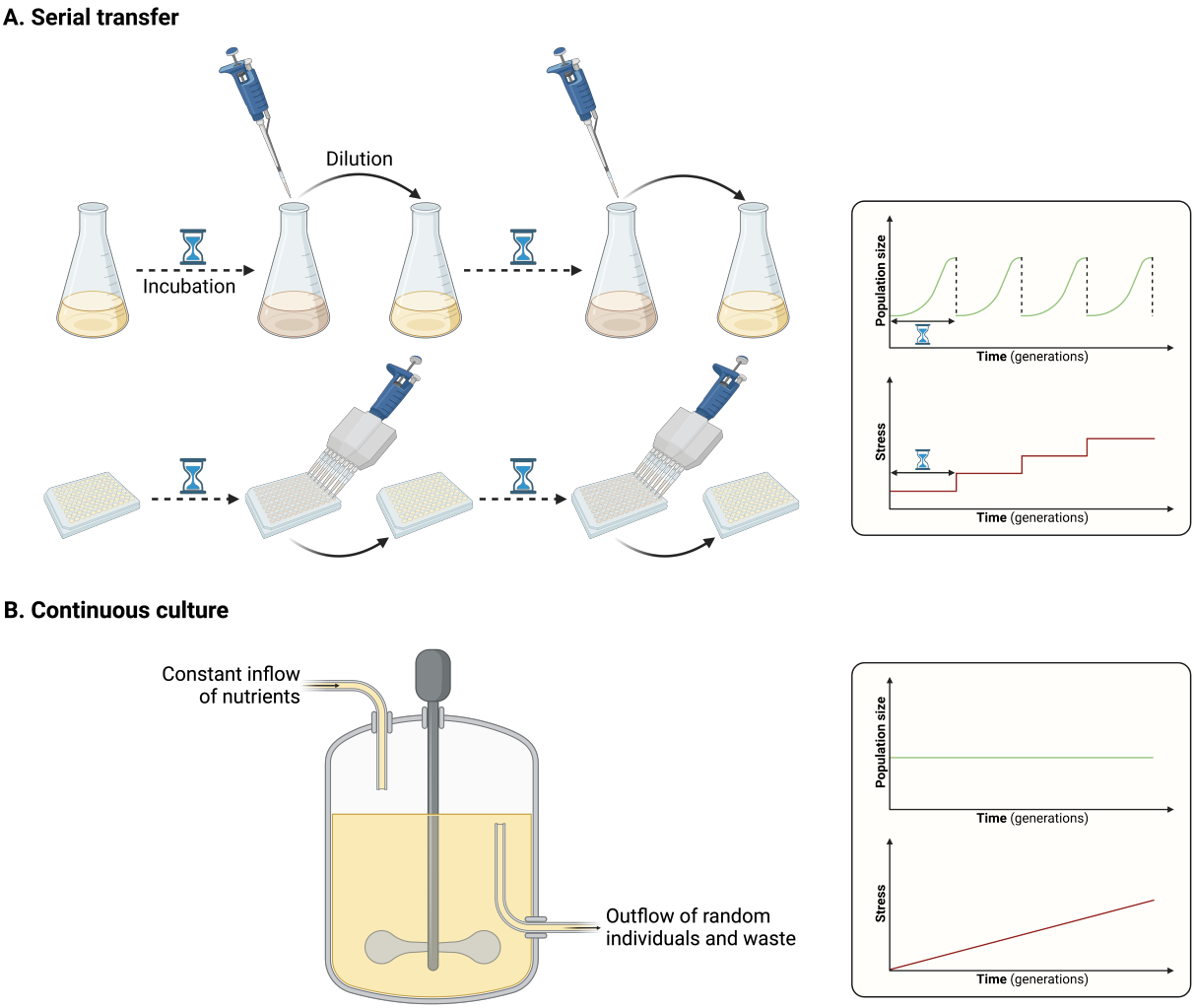

Figure 2: Overview of classical experimental evolution designs. The insets show the time-dependent changes in population size and external load in each setting. **A** In serial transfer experiments, a portion of the population is periodically transferred to fresh media and allowed to grow until the limiting nutrients are depleted. These experiments can be scaled to a large number of replicates, for example, when using 96-well plates. Typically, selection pressure (i.e., external stressors such as antibiotics) is increased incrementally at each dilution step. **B** Continuous culture systems include mechanisms for a constant supply of fresh medium and supplements that allow constant growth without large fluctuations in population size or growth phase. The morbidostat is often used in experiments where the bacterial response to increasing selection pressure is studied, because it allows the selection pressure to be continuously increased while keeping the population size as constant as possible. Figure adapted from [383, 386, 390] and created with BioRender.com.

Many experiments on microbial evolution use a constant, standardized environment that exerts a simple and moderate selection pressure on the organism. This often consists of the limited presence of a single

essential nutrient or the use of selection agents such as antibiotics. Usually, evolving populations need to be regularly diluted [383]. In this regard, sequential serial passages are among the most commonly used procedures because they are easy and simple to perform (e.g., [391, 392]). In a serial transfer experiment, the bacterial population is repeatedly transferred from one culture to a new culture over a series of generations, allowing a constant number of replications between dilution cycles (Figure 2A). Continuous culture systems (e.g., chemostat or morbidostat), on the other hand, allow the population to grow continuously in a container with a constant flow of nutrients, while the population size remains nearly constant (e.g., [393]) [386] (Figure 2B).

1.4 Bacterial virulence of high-risk clonal lineages

One Health-related high-risk clonal lineages are defined as causing difficult-to-treat and life-threatening infections affecting hosts from almost all branches of the tree of life [137]. These clonal lineages are known to combine MDR phenotypes with increased pathogenicity [135, 153, 394–396], which is the ability of a bacterial strain to cause infection in a host. However, pathogenicity must be considered in relation to the host, which responds to the pathogen with a defense strategy. The invader is detected early by pattern-recognition receptors and, depending on the host's immunocompetence, an immune response is elicited. The pathogen, in turn, has a repertoire of strategies to survive or persist in the conquered niche, called VFs. These virulence-associated attributes are usually defined as components of the pathogen whose loss specifically affects virulence but not viability (in nutrient-rich environments) [397]. These can be both structural elements and metabolites of the microorganism required for attachment to host cells, insensitivity to the host immune system (e.g., antiphagocytosis and protection from complement-mediated killing in serum), uptake of essential cofactors (especially iron ions) and limited nutrients, and production of toxins that can promote tissue invasion [160, 196].

In the narrowest sense, pathogenicity is the result of the dynamic relationship between host and pathogen, influenced by the ever-changing conditions of the pathogen, the host, and their environment. Virulence, on the other hand, is the quantitative capacity of the microorganism's pathogenic potency as determined by its genetic and physiological properties, and disease is the clinical outcome of the pathogen's virulence. While it is generally accepted that virulence-associated genes can be used to assess bacterial virulence, it is imperative to remember that virulence is highly context-dependent. It takes a multitude of steps (requiring VFs and physiological adaptations of the pathogen) to manifest the virulence of the pathogen in a given host. Detection of pathogenic bacteria in the environment, wildlife, and food (i.e., independent of clinically manifested disease) is difficult because reliable predictions of bacterial virulence are among the major limitations of WGS [398, 399]. Therefore, bioinformatics prediction relies on phenotypic characterizations through laboratory work. Certain *in vitro* and *in vivo* assays can be representative of the study of infection-related phenotypes mediated by specific factors. Below is a brief description of selected, clinically relevant virulence characteristics of One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae* that can be studied using such *in vitro* or *in vivo* assays (Figure 3). It should be noted, however, that a comprehensive review of all potential mechanisms contributing to the virulence of these clones is beyond the scope of this paragraph. Moreover, different

mechanisms may be mutually dependent and lead to different clinical manifestations depending on various circumstances, such as host immunocompetence and genetic makeup of the pathogen.

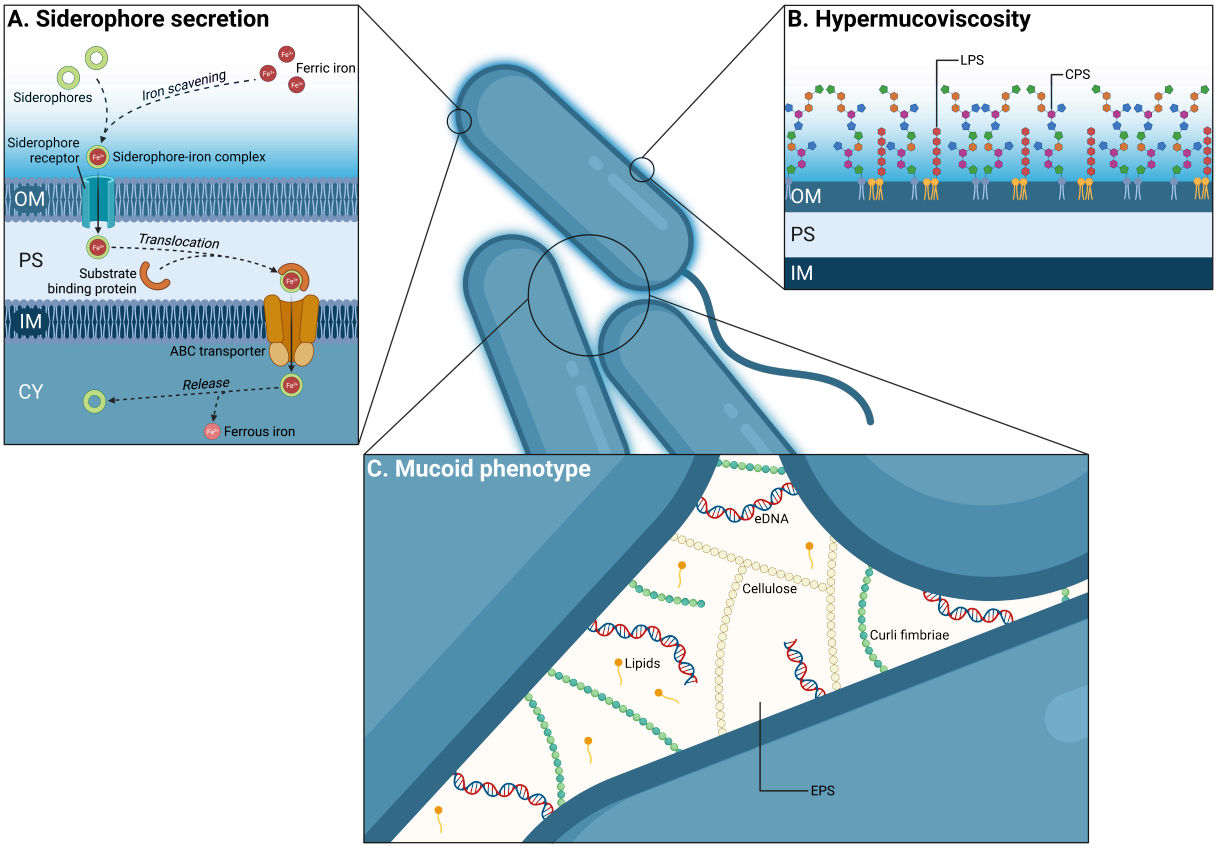

Figure 3: Selected bacterial virulence-associated characteristics of One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae*. **A** Siderophore secretion is the process by which bacteria produce and secrete small, high-affinity iron chelators, called siderophores, into their environment, allowing them to survive and grow in the presence of iron deficiency, such as in host tissues. Siderophores bind tightly to ferric iron (Fe^{3+}) in the environment and form a siderophore-iron complex that is recognized and taken up by specific siderophore receptors on the outer membrane of the bacterial cell. After internalization and energy-dependent translocation across the periplasmic space and inner membrane, the siderophore-iron-complex is degraded by reduction of the coordinated ferric iron to ferrous iron (Fe^{2+}), and the iron is released into the cytosol for cellular metabolism. **B** Hypermucoviscosity is a bacterial phenotype resulting from the overproduction of capsule polysaccharides that lead to a particularly thick and sticky layer of mucus with increased viscosity. **C** The expression of the mucoid phenotype results from the excessive production of an extracellular polymeric substance. This substance is a complex mixture of biopolymers, including polysaccharides, proteins, nucleic acids, and lipids, that are produced by the bacteria and then excreted into the environment. The resulting complex matrix plays a crucial role in the adhesion of bacteria to surfaces and in the formation and stability of biofilms. It provides structural support for the biofilm and acts as a barrier, protecting the bacteria from external stresses such as antibiotics, detergents, and desiccation. Figure adapted from [400–403] and created with BioRender.com. CY, cytosol. IM, inner membrane. OM, outer membrane. PS, periplasmic space. CPS, capsule polysaccharide. EPS, extracellular polymeric substance. LPS, lipopolysaccharide. eDNA, extracellular DNA.

Pathogens rely on external resources to escape, survive, and persist inside the host. In particular, at infection sites, the availability of free extracellular ferric iron (Fe^{3+}) to the invading pathogen is severely limited due to the high demand of the bacterial invader and iron deprivation by the host immune defense system [404]. The latter has the intention of starving bacteria. To overcome this limitation, siderophores, which are low molecular weight molecules (0.5–1.5 kDA) that chelate Fe^{3+} with high affinity [405], are synthesized by bacteria and released into the environment [404, 406] (Figure 3A). Once an Fe^{3+}-siderophore complex is formed, it is internalized by specific energy-dependent carrier proteins on the cell surface [407, 408]. In the cytosol, the coordinated ferric iron is reduced to ferrous iron (Fe^{2+}) by biological reducing agents such as NADH. The resulting Fe^{2+}-siderophore complex is kinetically and thermodynamically less stable, so ferrous iron is released. The free siderophore is then recycled and re-released into the environment [405, 408]. Clinically, extensive siderophore secretion is closely

associated with urinary tract and wound infections as well as pneumonia [409–415], so siderophore receptors expressed on the bacterial surface (e.g., the receptor for aerobactin IutA) may be used as potential vaccine antigens [416] and siderophore conjugates can be used as a promising shuttle of antibiotics into the bacterial cell [417, 418].

The invading pathogen not only faces nutrient deprivation, but must also defend itself from the host immune response. One important way pathogens evade the host immune response is by forming a physical barrier that prevents host immune cells and molecules, such as phagocytes, complement proteins, and antibodies, from reaching and attacking bacterial cells. There is growing evidence that hypermucoviscosity or the expression of a mucoid phenotype are often associated with highly virulent pathogens. Although the terms “hypermucoviscosity” and “mucoid phenotype” are often used interchangeably and associated with a positive string test [256], they are related but distinct phenomena. Hypermucoviscosity refers specifically to the increased viscosity of bacterial pathogens due to the overproduction of capsular polysaccharides [258] (Figure 3B), which is common in *K. pneumoniae* (predominantly hvKp [244]) but can also occur in pathogenic *E. coli* strains [419–421]. However, capsule thickness does not necessarily correlate with hypermucoviscosity [258]. Instead, hypermucoviscosity appears to be more related to the composition of monosaccharides within the capsule polysaccharide (CPS) chain [422–424]. Specifically, hvKp strains are often associated with capsule types (K) that do not contain mannose monomers (e.g., K2 or K20), which are used as recognition structures by macrophages [425]. Indeed, the overproduction of CPS, associated with the hypermucoviscous phenotype of *K. pneumoniae*, impedes cell attachment but confers the ability to evade neutrophil-mediated phagocytosis [375, 426–429] and human defensin-mediated bactericidal activity [430]. *K. pneumoniae* strains exhibiting hypermucoviscosity are clinically associated with invasive infections such as pyogenic liver abscesses, necrotizing fasciitis, septic thromboembolism, and epidural abscesses [246, 431–434].

In contrast, the mucoid phenotype refers to the excessive production of a mucilaginous, viscous substance called extracellular polymeric substance (EPS) that results in shiny, raised, opaque, blob-like colonies on agar plates (Figure 3C). EPS is usually a mixture of polysaccharides, proteins, and other molecules, but its composition and their physiological significance varies considerably across species [435, 436]. For example, *E. coli* strains with a mucoid phenotype have a predilection for the urinary tract and cause corresponding infections [437], whereas predominantly hypomucoid *K. pneumoniae* strains cause urinary tract infections [375]. This phenomenon may be due to the fact that EPS of *E. coli* contributes to attachment to the cell surface of endothelial cells in the urinary tract and to biofilm formation [438–441].

Biofilms are bacterial communities that attach to a surface and are surrounded by self-produced EPS. Biofilm formation is a defense mechanism of pathogens that complicates the treatment of infections and can lead to chronic infections [442]. The idea of a life cycle of biofilms was developed through research on *Pseudomonas aeruginosa* (*P. aeruginosa*) in a laboratory setting and has since been used to describe biofilms in general [443]. However, it is important to note that biofilm formation is a complex process and can differ depending on the bacterial species involved [444]. In *E. coli*, biofilm formation is usually initiated by *csg* (curli-associated sortase-generated) genes that regulate the biosynthesis of curli

amyloid fibers [440, 445]. After their initial attachment, the bacteria cells begin to grow and multiply, forming a dense community. As they continue to grow, the bacteria produce curli fibers that are cross-linked by sortase enzymes, forming a strong, sticky, three-dimensional matrix with various layers and channels for transport of nutrients and waste [444]. This EPS matrix holds the bacterial cells together, protecting them from the environment and making it difficult for antibiotics and the immune system to kill them [446]. Eventually, the biofilm may begin to disintegrate and disperse, releasing individual planktonic cells into the environment, which can then form new biofilms or infect other hosts [443].

Although considerable efforts have been made in bioinformatics and *in vitro* characterization, the *in vivo* animal model remains the gold standard for determining virulence. In recent years, larvae of the greater wax moth *Galleria mellonella* (*G. mellonella*) have proven to be a reliable and cost-effective model [261, 447, 448], although some authors have argued that only the mouse model can accurately assess virulence of *E. coli* and *K. pneumoniae* [449]. Nevertheless, the use of the *G. mellonella* model is consistent with the principles of “3R” (Replacement, Reduction, and Refinement) in animal studies. These principles are enshrined in national and international legislation, aim to replace vertebrate infection models, and reduce the number of mammals used in research [450]. Furthermore, because *G. mellonella* is an invertebrate, it is not subject to animal welfare laws and ethical guidelines. This, together with its physiological and immunological characteristics, makes it an ideal model to study the virulence of bacterial pathogens and the efficacy of antibiotics [448]. Most importantly, the larvae are amenable to incubation at 37 °C, which corresponds to the body temperature of the human host. In addition, the immune system of *G. mellonella* shares many similarities with the innate immune response of mammals [451–453]. As far as virulence determination is concerned, the absence of adaptive immunity can be considered an advantage for research, as the model allows the study of host-pathogen interactions and related mechanisms of innate immunity without the influence of adaptive responses [454].

2 AIMS OF THIS THESIS

This work explores the drivers and factors that contribute to the occurrence, emergence, and adaptation of successful One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae* in different contexts at the genotypic and phenotypic levels. It includes (i) the analysis of genomic survey data of *E. coli* isolates from ecologies in sub-Saharan Africa (**Publications 1** and **2**), (ii) the application of functional genomics and phenotyping techniques to characterize bacterial virulence and assess its clinical relevance in the context of a food-borne *E. coli* strain (**Publication 3**), and (iii) the investigation of evolutionary pathways promoting the development of resistance to a novel drug combination and compensatory mechanisms in a *K. pneumoniae* strain (**Publication 4**).

3 RESULTS

3.1 Publication 1

The study "**Flies from a tertiary hospital in Rwanda carry multidrug-resistant Gram-negative pathogens including extended-spectrum β-lactamase-producing *E. coli* sequence type 131**", analyzed houseflies collected in the University Teaching Hospital of Butare (Rwanda) for the occurrence and characteristics of MDR bacteria. Investigation revealed that among the 42 houseflies randomly captured in fly traps, half carried Gram-negative bacteria that combined AMR with genotypically high bacterial virulence, including the high-risk *E. coli* ST131, ST167, ST410, and ST617. All identified bacterial strains were MDR but not resistant to carbapenems or colistin [113].

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Contributions (according to the CRediT taxonomy [455])

Stefan E. Heiden	data curation, formal analysis, investigation, software, visualization
Mathis S. E. Kurz	formal analysis, investigation
Jürgen A. Bohnert	formal analysis
Claude Bayingana	formal analysis, resources
Jules M. Ndoli	formal analysis, resources
Augustin Sendegeya	formal analysis, resources
Jean B. Gahutu	formal analysis, resources
Elias Eger	data curation, formal analysis, investigation
Frank P. Mockenhaupt	conceptualization, project administration, supervision, funding acquisition, resources, writing – original draft
Katharina Schaufler	conceptualization, project administration, supervision, funding acquisition, validation, writing – original draft, review, and editing

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Antimicrobial Resistance and Infection Control

SHORT REPORT Open Access

Flies from a tertiary hospital in Rwanda carry multidrug-resistant Gram-negative pathogens including extended-spectrum beta-lactamase-producing *E. coli* sequence type 131

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Abstract

Multidrug-resistant gram-negative (MRGN) bacteria are a serious threat to global health. We used genomics to study MRGN obtained from houseflies in a tertiary Rwandan hospital. Our analysis revealed a high abundance of different MRGN including *E. coli* pathogenic lineage ST131 suggesting the important role of flies in disseminating highly virulent pathogens in clinical settings and beyond.

Keywords: MRGN, Vector flies, Virulence

Text

Multidrug-resistant gram-negative (MRGN) bacteria include *Escherichia* (*E.*) *coli*, *Klebsiella* spp., *Enterobacter (E.) cloacae*, *Acinetobacter* spp., and *Pseudomonas* (*P.*) *aeruginosa*, and others, and cause a variety of severe infections like diarrhea, pneumonia, sepsis, endocarditis and urinary tract infection (UTI). Studies estimate 700.000 fatalities caused by antibiotic-resistant pathogens each year with increasing numbers [1]. In addition to their common occurrence as nosocomial pathogens, MRGN have been frequently found in livestock and the environment. Flies have only recently come into spotlight as carriers of resistant bacteria, and their major route of colonization stems from walking on contaminated surfaces [2]. The detection of antibiotic-resistant *E. coli* from flies captured in a livestock facility was thus unsurprising [3]. Another study has shown that houseflies from hospitals in the UK carried different bacteria resistant to antibiotics [4]. We investigated if houseflies captured in a tertiary hospital in Rwanda carried clinically relevant MRGN pathogens. In African hospital settings, where hygienic conditions may be suboptimal [5], flies might function as underestimated vectors for the distribution of antibiotic-resistant bacteria.

We examined 42 flies randomly captured in fly traps within 4 weeks in a tertiary hospital in Rwanda in 2014 [5]. Sampling locations included surgery, gynecologic and other wards (Fig. 1a/b). Because we initially focused on cefotaxime-resistant representatives, bacteria carried by flies were first enriched in tryptic soy broth and then cultured on chromogenic agar (CHROMagar-ESBL, Mast Diagnostica, Germany) supplemented with 2 μg/mL cefotaxime. For "extended-spectrum beta-lactamase (ESBL)-positive" colonies, ESBL and/or ampicillinase (AmpC) production was verified (ESBL-AmpC-Detection Test, Mast Diagnostica [6]), and strains positive for AmpC only were excluded. After preselecting putative strains of *E. coli, Klebsiella* spp., *Enterobacter* spp., *Acinetobacter* spp., *P. aeruginosa, Citrobacter* spp., and *Raoultella* spp., we confirmed the bacterial species using MALDI-TOF (Bruker Daltonics, Germany). Additional phenotypic resistance screening was performed on the VITEK 2 system (bioMérieux, France) and for colistin resistance on 96-well microtiter plates investigating minimal

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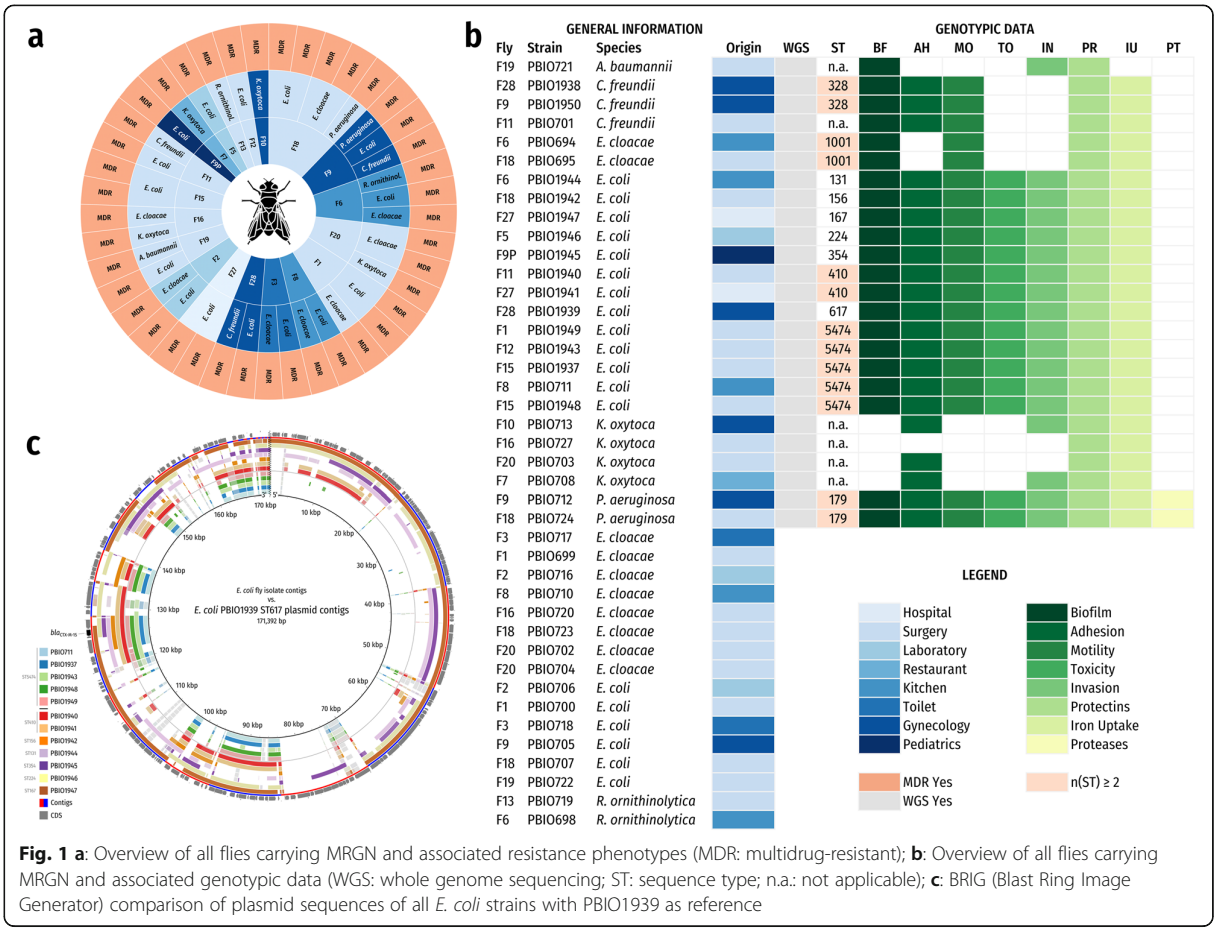

Fig. 1 **a**: Overview of all flies carrying MRGN and associated resistance phenotypes (MDR: multidrug-resistant); **b**: Overview of all flies carrying MRGN and associated genotypic data (WGS: whole genome sequencing; ST: sequence type; n.a.: not applicable); **c**: BRIG (Blast Ring Image Generator) comparison of plasmid sequences of all *E. coli* strains with PBIO1939 as reference

inhibitory concentrations in triplicates. Randomly selected strains (Fig. 1a/b) were whole genome sequenced (WGS) on an Illumina MiSeq/NovaSeq 6000 (Eurofins Genomics Europe Sequencing GmbH, Germany). Raw reads were quality-trimmed, adapter-trimmed and contaminant-filtered using BBDuk from BBTools (https://sourceforge.net/projects/bbmap/files/BBMap_38.41.tar.gz/download). After *de-novo* assembly using shovill/SPAdes (https://github.com/tseemann/shovill/archive/v1.0.4.tar.gz; http://cab.spbu.ru/files/release3.13.1/SPAdes-3.13.1.tar.gz) and Velvet, draft genomes were polished by mapping all trimmed reads back to the contigs with bwa (https://github.com/lh3/bwa/releases/download/v0.7.17/bwa-0.7.17.tar.bz2) and calling variants with Pilon (https://github.com/broadinstitute/pilon/releases/download/v1.23/pilon-1.23.jar). *E. coli* plasmid sequences of PBIO711 and PBIO1939 were manually extracted using similarity searches (BLASTn Megablast) against the NCBI nucleotide collection for visualization in BRIG (Blast Ring Image Generator) (https://sourceforge.net/projects/brig/files/dev/BRIG-0.95-dev.0004.zip/download). Sequence type (ST), antibiotic resistance/virulence gene and single-nucleotide polymorphism (SNP) detection was carried out using mlst, abricate, and snippy (https://github.com/tseemann/mlst/archive/v2.16.1.tar.gz; https://github.com/tseemann/abricate/archive/v0.8.11.tar.gz; https://github.com/tseemann/snippy/archive/v4.4.1.tar.gz). We inferred a core SNP phylogeny for ST5474. Alignments were filtered for recombinations using Gubbins (https://github.com/sanger-pathogens/gubbins/archive/v2.3.4.tar.gz) and core SNPs extracted using snp-sites (1745 sites; https://github.com/sanger-pathogens/snp-sites/archive/v2.4.1.tar.gz). A maximum likelihood tree was inferred with RAxML-NG (https://github.com/amkozlov/raxml-ng/releases/download/0.9.0/raxml-ng_v0.9.0_linux_x86_64.zip) using GTR + G. The best-scoring maximum likelihood tree was midpoint-rooted and visualized in FigTree (https://github.com/rambaut/figtree/releases/download/v1.4.4/FigTree.v1.4.4.zip).

Overall 48% (20/42) of flies carried antibiotic-resistant bacteria. Thirty-six percent (15/42) carried ESBL-producing *E. coli*, 19% (8/42) *E. cloacae*, 9% (4/42) *K.*

oxytoca, 7% (3/42) *C. freundii*, 4% (2/42) *R. ornithinolytica*, 4% (2/42) *P. aeruginosa*, and 2% (1/42) *A. baumannii*. Twelve flies (29%) carried more than one antibiotic-resistant bacterial genus of which three (F6, F9 and F18) carried three different pathogens (Fig. 1a/b).

All strains were phenotypically multidrug-resistant and thus termed MRGN (Fig. 1a), however they were not resistant to carbapenems or colistin. WGS revealed carriage of different antimicrobial resistance genes such as $bla_{\text{CTX-M-15}}$, *aac* [3]-*IIa*, and *tet*(A)/(B) (Table S1). Eight different STs were observed including ST131 and ST410 (Fig. 1b). Interestingly, these represent international high-risk clonal lineages [7, 8], which combine antimicrobial resistance with high-level virulence. The ST131 strain harbored ten resistance genes and 31 virulence-associated genes including the *pap* operon linked to UTI [9] (Table S1).

In addition, we observed five *E. coli* strains of ST5474, which is a ST recently associated with enterotoxigenic *E. coli* (ETEC) causing diarrhea [10]. This might point towards fly pollution through stool-contaminated surfaces, possibly through a common source. However, note that we did not detect the ETEC-defining heat-labile and/or heat-stable toxins. Our phylogenetic analysis suggested clonality among our five ST5474 strains (1–9 SNPs/aligned Mbp), and similarity to five publicly available ST5474 genomes (178–560 SNPs/aligned Mbp) (Figure S2).

Three *E. coli* strains (PBIO1939, PBIO1940 and PBIO1941), which did not only originate from individual flies captured in different wards but belonged to two different clonal lineages (ST410 and ST617), carried similar resistance genes (Table S1), however they differed in their overall plasmid content (Fig. 1c).

The two *P. aeruginosa* genomes contained several previously described virulence features mandatory for severe invasive infections including flagella, the type III secretion system, type IV pili, as well as toxins and proteases. The *A. baumannii* genome carried virulence genes associated with serum survival and invasion (phospholipase PLC) (Table S1). Overall, all analyzed genomes showed high virulence potentials (Fig. 1b).

Our results demonstrate that half of the flies in this tertiary hospital in Rwanda carried virulent MRGN pathogens including the pathogenic clonal *E. coli* lineage ST131. High pre-admission and even higher discharge rates at this facility [5] may suggest that a) patients and caregivers were the source of MRGN for the flies and b) that flies play a role in the transmission of antimicrobial-resistant pathogens within clinics and in mirroring the burden of antimicrobial resistance [4] at that time. Even though the actual transmission of MRGN bacteria through flies to humans awaits verification, respective modelling results point strongly into this direction [11].

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13756-020-0696-y.

Additional file 1: Table S1. Results based on whole genome sequence analysis. Abbreviations: BLA: beta-lactams (incl. ampicillin, piperacillin, cefuroxime, cefpodoxime, cefotaxime, ceftazidime); GEN: gentamicin; CIP: ciprofloxacin/moxifloxacin; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; ST: sequence type; resistance, virulence and plasmid genes are based on the abricate (https://github.com/tseemann/abricate) abbreviations using the databases Resfinder, ARG-ANNOT, CARD, NCBI Bacterial Antimicrobial Resistance Reference Gene Database, PlasmidFinder, VFDB, and Ecoli_VF.

Additional file 2: Figure S2. Phylogenomic tree of five *E. coli* sequence type (ST) 5474 fly isolates (strain names colored according to Fig. 1c) and publicaly available WGS data of five ST5474 strains (raw read accession nos.; black).

Abbreviations

ESBL: Extended-spectrum beta-lactamase; ETEC: Enterotoxigenic *E. coli*; MRGN: Multidrug-resistant gram-negative; SNP: Single-nucleotide polymorphism; ST: Sequence type; UTI: Urinary tract infection; WGS: Whole genome sequencing

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Authors' contributions

KS and FPM designed and drafted the manuscript. Experiments were performed by SEH, EE and MSEK. JB, CB, JMN, AS and JBG helped analyzing the results. JB, CB, JMN, AS and JBG helped in proofreading and editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB36565 (https://www.ebi.ac.uk/ena/data/view/PRJEB36565).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

3.2 Publication 2

The study "**Circulation of extended-spectrum β-lactamase-producing *Escherichia coli* of pandemic sequence types 131, 648, and 410 among hospitalized patients, caregivers, and the community in Rwanda**", investigated whether clinically relevant high-risk *E. coli* lineages were present in the University Teaching Hospital of Butare (Rwanda) and surrounding locations. Based on phylogenetics and in-depth genotyping, we analyzed the underlying characteristics and putative transmission dynamics of both bacterial pathogens and resistance plasmids. We examined 120 ESBL-producing *E. coli* strains derived from rectal swabs of hospitalized patients and their caregivers, as well as community members and livestock. We also included 13 genomes of strains derived from houseflies caught in different wards of the Rwandan hospital (s. Publication 1).

Our analysis revealed that the most abundant STs were ST131, ST354, ST405, ST410, and ST648, which accounted for approximately 50% of all strains. Phylogenetics showed that the strains were distributed among six different phylogroups, with the majority of strains belonging to phylogroup A. All strains were genotypically MDR. The predominant ESBL gene was *bla*$_{\text{CTX-M-15}}$, inherited by 87.5% of all strains. In addition, strains belonging to the five predominant STs carried several virulence-associated genes mainly associated with adherence, antiphagocytosis, biofilm formation, invasion, iron uptake, and bacterial secretion. Genes encoding efflux pumps were also frequently found in our samples. Most strains carried plasmids containing IncFIB, followed by IncFIA and IncFII. The plasmid backbones of the strains studied showed striking similarities, and similar resistance plasmids were found in strains of different sequence types, which indicates a possible transfer of mobile genetic elements [112].

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Circulation of Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* of Pandemic Sequence Types 131, 648, and 410 Among Hospitalized Patients, Caregivers, and the Community in Rwanda

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Multi-drug resistant (MDR), gram-negative *Enterobacteriaceae*, such as *Escherichia coli* (*E. coli*) limit therapeutic options and increase morbidity, mortality, and treatment costs worldwide. They pose a serious burden on healthcare systems, especially in developing countries like Rwanda. Several studies have shown the effects caused by the global spread of extended-spectrum beta-lactamase (ESBL)-producing *E. coli*. However, limited data is available on transmission dynamics of these pathogens and the mobile elements they carry in the context of clinical and community locations in Sub-Saharan Africa. Here, we examined 120 ESBL-producing *E. coli* strains from patients hospitalized in the University Teaching Hospital of Butare (Rwanda), their attending caregivers as well as associated community members and livestock. Based on whole-genome analysis, the genetic diversification and phylogenetics were assessed. Moreover, the content of carried plasmids was characterized and investigated for putative transmission among strains, and for their potential role as drivers for the spread of antibiotic resistance. We show that among the 30 different sequence types (ST) detected were the pandemic clonal lineages ST131, ST648 and ST410, which combine high-level antimicrobial resistance with virulence. In addition to the frequently found resistance genes $bla_{CTX-M-15}$, *tet*(34), and *aph(6)-Id*, we identified *csg* genes, which are required for curli fiber synthesis and thus biofilm formation. Numerous strains harbored multiple virulence-associated genes (VAGs) including *pap* (P fimbriae adhesion cluster), *fim* (type I fimbriae) and *chu* (Chu heme uptake system). Furthermore, we found phylogenetic relationships among strains from patients and their caregivers or related community members and animals, which indicates transmission of pathogens. Also, we

demonstrated the presence and potential transfer of identical/similar ESBL-plasmids in different strains from the Rwandan setting and when compared to an external plasmid. This study highlights the circulation of clinically relevant, pathogenic ESBL-producing *E. coli* among patients, caregivers and the community in Rwanda. Combining antimicrobial resistance with virulence in addition to the putative exchange of mobile genetic elements among bacterial pathogens poses a significant risk around the world.

Keywords: ESBL—*E. coli*, whole-genome sequencing, Rwanda, virulence factors, phylogenetic analysis

INTRODUCTION

The versatility of *Escherichia coli* (*E. coli*) is based on the diversity of genetic substructures within this species (Whittam et al., 1983). In addition to commensal strains, which are an essential part of the non-anaerobic intestinal microflora of humans, other mammals and birds, pathogenic variants occur. The dissimilarity of these pathotypes depends also on their virulence attributes, resulting in a wide range of pathologies in both humans and animals. The intestinal pathogenic *E. coli* (InPEC) express characteristic virulence factors that allow to adhere and invade intestinal cells, causing specific enteric and diarrheal diseases. While InPEC are obligate pathogens, extraintestinal pathogenic *E. coli* (ExPEC) are part of the intestinal microbiome but exhibit a heterogeneous composition of virulence factors to colonize niches such as the urinary tract (Kaper et al., 2004). They can thus cause infections in almost any organ or non-intestinal site, regardless of the state of the host's immune system (Russo and Johnson, 2000). However, a strict differentiation of pathogenic and commensal *E. coli* is difficult, provided by their rapid geno- and phenotypic adaptation to changing environmental conditions, for example through horizontal gene transfer (Pallen and Wren, 2007). Despite the plasticity of the genome, phylogenetic studies have shown some clonality within the population structure of *E. coli*, from which seven distinct phylogenetic groups were derived (Jaureguy et al., 2008; Touchon et al., 2009; Clermont et al., 2013). Usually, commensal strains and obligatory pathogens belong to the phylogroups A and B1, whereas strains with extended virulent attributes (mainly ExPEC) are part of the phylogroups B2, D, and F, with the latter as a sister group of B2 (Escobar-Páramo et al., 2004; Clermont et al., 2013). Multi-locus sequence typing (MLST) allows additional classification and several phylogenetic studies suggest the spread of pandemic high-risk clonal lineages including primarily sequence type (ST) 131 (Nicolas-Chanoine et al., 2008; Ewers et al., 2010; Hussain et al., 2012), ST648 (Ewers et al., 2014; Schaufler et al., 2019), ST410 (Schaufler et al., 2016b; Zurita et al., 2020), putatively ST405 (Manges et al., 2019), and others.

The management of zoonotic infections caused by antibiotic-resistant bacteria has become a multidisciplinary challenge for all modern healthcare systems and is nowadays often approached in a holistic One Health context. Bacterial pathogens spread through direct contact among humans and animals, indirectly by (environmental) pollution and also through non-living and living vectors (Rahman et al., 2020). One example for the latter are houseflies, which have been demonstrated to carry antibiotic-resistant pathogens including extended-spectrum beta-lactamases-(ESBL)-producing *E. coli* (Rahuma et al., 2005; Heiden et al., 2020b; Tufa et al., 2020) non-susceptible to third-generation cephalosporins (e.g., cefotaxime) and monobactams (e.g., aztreonam) (Bevan et al., 2017). Notably, ESBL enzyme production is often accompanied by cross- and co-resistances (Cantón and Coque, 2006; Hidron et al., 2008; Pitout, 2012) resulting in multi-drug resistant (MDR) representatives (Beceiro et al., 2013).

Both pandemic clonal lineages including the aforementioned ST131, ST648 and others, as well as mobile genetic elements (i.e., ESBL-plasmids) drive the spread of antibiotic resistance and virulence-associated genes (VAGs) (Cantón and Coque, 2006). Interestingly, previous studies suggest that ESBL-plasmid carriage does not ineluctably reduce bacterial fitness, which seems particularly true for specific clonal lineages (McNally et al., 2016; Schaufler et al., 2016a; Ranjan et al., 2018; Monárrez et al., 2019; Schaufler et al., 2019). The combination of MDR with high-level bacterial virulence and fitness leads to the emergence of these pandemic, high-risk clonal lineages and subsequently contributes to treatment failures, increasing morbidity, and mortality (Melzer and Petersen, 2007; Schwaber and Carmeli, 2007; Tumbarello et al., 2007; Beceiro et al., 2013; Schaufler et al., 2019; Heiden et al., 2020a).

The One Health concept—addressing human, animal and environmental health—encounters some challenges, especially in low-income countries like Sub-Saharan Africa/Rwanda. On the one hand, the lack of surveillance systems may result in inadequate establishment and implementation of hygienic strategies and therapy guidelines (Muvunyi et al., 2011; Ntirenganya et al., 2015; Carroll et al., 2016). On the other hand, uncontrolled over-the-counter sale of partially counterfeit and substandard antibiotic drugs (Kayumba et al., 2004; Carroll et al., 2016) as well as close human-livestock contact and household crowding might contribute to the broad occurrence and interspecies transmission of MDR bacteria in Sub-Saharan Africa.

This study aimed to investigate whether (i) ESBL-producing *E. coli* circulate among patients, caregivers, the community, and animals in Rwanda, (ii) some of these belong to pandemic high-risk clonal lineages and how they are phylogenetically related, (iii) they demonstrate virulence-associated features, (iv) their mobile genetic elements contribute to the spread of antibiotic resistance.

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* strains investigated in this study were sampled over a time period of 8 weeks at the University Teaching Hospital of Butare (Rwanda) in 2014 (previously described by Kurz et al., 2017). Rectal swabs (Sarstedt AG & Co. KG, Nümbrecht, Germany) were collected from patients and caregivers at admission and discharge as well as from several community members and animals. Each patient had their own caregiver, who were usually relatives accompanying the patient upon admission. They stayed in the patient's room and were involved in personal care of the patient and food preparation. This is a common practice in African hospitals (Hoffman et al., 2012; Ugochukwu, 2013). Sample groups consisting of a patient and related caregiver, and associated family members, neighbors and/or pets were included in the same study-ID. The samples were plated onto chromogenic agar (CHROMagar-ESBL, Mast Diagnostica GmbH, Reinfeld, Germany) supplemented with 2 μg/mL cefotaxime (Cayman Chemical Company, Ann Arbor, United States) and incubated at 37°C. For putative ESBL-positive colonies, the production of ESBL and/or ampicillinase (AmpC) was verified (ESBL-AmpC-Detection Test, Mast Diagnostica GmbH, Reinfeld, Germany) and all strains positive for AmpC only were excluded. The strains were stored at −80°C in LB broth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) supplemented with 20% (V/V) glycerol (Merck KGaA, Darmstadt, Germany). Originally, we have obtained overall 289 ESBL-producing *E. coli* strains (from patients, caregivers, community members, and animals), with 120 selected strains (based on related study-IDs) that were whole-genome sequenced. Additionally, flies caught with fly traps at different wards of the hospital examined in a previous study (Heiden et al., 2020b) were partly included in this study (**Supplementary Table 1**).

Whole-Genome Sequencing

One single *E. coli* colony was cultured in LB broth supplemented with 2 μg/mL cefotaxime overnight and the total DNA was extracted using the MasterPure™ DNA Purification Kit for Blood, Version II (Lucigen, Middleton, United States) according to the manufacturer's instructions. DNA was purity-controlled and quantified using NanoDrop™ 2000 (Thermo Fisher Scientific Inc., Waltham, United States). WGS was performed in collaboration with LGC (LGC Genomics GmbH, Berlin, Germany) with 150 bp paired-end-reads using Illumina NextSeq 500/550 V2.

Genomic Analysis

Raw reads were quality-trimmed, adapter-trimmed and contaminant-filtered using BBDuk from BBTools v. 38.86[1]. After *de novo* assembly (at a maximum coverage of 100×) using shovill v. 1.1.0[2] in combination with SPAdes v. 3.14.1 (Bankevich et al., 2012), draft genomes were polished by mapping all trimmed reads back to the contigs with bwa v. 0.7.17 (Li and Durbin, 2009), processing SAM/BAM files marking optical duplicates with Samtools v. 1.10 (Li et al., 2009) and calling variants with Pilon v. 1.23 (Walker et al., 2014) (**Supplementary Table 2**).

Plasmid sequences of all strains were manually extracted using similarity searches (BLASTN Megablast) against the NCBI nucleotide collection for visualization in BRIG v. 0.95-dev.0004 (Alikhan et al., 2011). The *in silico* MLST, antibiotic resistance/virulence gene and single-nucleotide polymorphism (SNP) detection was carried out using mlst v. 2.19.0[3], ABRicate v. 1.0.0[4], and snippy v. 4.6.0[5]. We inferred core SNP phylogenies for pandemic sequence types. Alignments were filtered for recombinations using Gubbins v. 2.4.1 (Croucher et al., 2015) and core SNPs extracted using snp-sites v. 2.5.1 (Page et al., 2016) [core SNP sites filtered out: 1,219 (ST131); 0 (ST354); 5,370 (ST405); 2,940 (ST410); 3,564 (ST648)]. The final core SNP alignments contained 208 (ST131; reference: PBIO440), 20 (ST354; reference: PBIO388), 177 (ST405; reference: PBIO397), 154 (ST410; reference: PBIO289), and 135 (ST648; reference: PBIO368) sites (**Supplementary Table 3**). Maximum likelihood trees were inferred with RAxML-NG v. 1.0.0 (Kozlov et al., 2019) using GTR + G (discrete GAMMA model of rate heterogeneity with 4 categories) and searching from 500 random and 500 parsimony-based starting trees. The best-scoring maximum likelihood tree supplemented with support values from 1,000 non-parametric bootstrap replicates was midpoint-rooted and visualized with iTOL v. 5.7 (Letunic and Bork, 2019). To assess the population structure of all available genomes, we inferred an additional phylogeny constructed with JolyTree v 2.0.19092ac (Criscuolo, 2019) of all 120 strains from this study and 13 housefly isolates previously published (Heiden et al., 2020b). A synteny plot comparing ST38 chromosome- and plasmid-derived contigs was created with genoPlotR v 0.8.9 (Luan and Li, 2004).

Minimum Inhibitory Concentration of Colistin

When the genotype was positive for colistin resistance (presence of mcr genes), we evaluated the resistance phenotype by determining the minimum inhibitory concentration (MIC) using MICRONAUT MIC-Strip Colistin (Merlin Diagnostika GmbH, Bornheim, Germany) according to the manufacturer's instructions and interpreted the results according to the published breakpoints of EUCAST (The European Committee on Antimicrobial Susceptibility Testing, 2021). Experiments were performed thrice.

RESULTS

Phylogenetic Grouping and Multi-Locus Sequence Typing

The largest fraction of the 120 ESBL-producing *E. coli* originated from rectal swabs of patients (50.8%; 61/120), followed by caregivers (38.3%; 46/120), neighbors (5.0%; 6/120), family members (4.2%; 5/120), and animals (1.7%; 2/120) (**Figure 1**

[1]https://sourceforge.net/projects/bbmap/

[2]https://github.com/tseemann/shovill/

[3]https://github.com/tseemann/mlst/

[4]https://github.com/tseemann/abricate/

[5]https://github.com/tseemann/snippy/

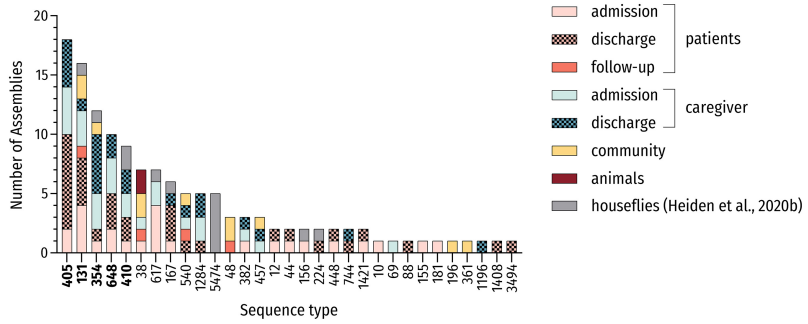

FIGURE 1 | MLST-based distribution of investigated strains. Distribution of all investigated strains including genomes of ESBL-producing *E. coli* carried by houseflies (Heiden et al., 2020b; n = 133) and associated sequence types (STs). The five most prevalent STs are in bold.

and **Supplementary Table 1**). The majority of strains belonged to phylogroup A (30.8%; 37/120), which usually comprises commensal strains. The remaining strains were distributed among the phylogroups D (21.7%; 26/120), F (20.0%; 24/120), and B2 (14.2%; 17/120) as well as phylogroups B1 (6.7%; 8/120) and C (6.7%; 8/120), based on Clermont's revised *E. coli* phylotyping method (Clermont et al., 2013).

Multi-locus sequence typing (MLST) from genomic data revealed 30 different STs. In total, sixty percent (18/30) of identified STs were present more than once and 10 or more strains belonged to 4 main STs (13.3%; 4/30) including the high-risk ST131 and ST648 *E. coli* lineages. These most frequently encountered STs were ST405 (15.0%; 18/120), ST131 (12.5%, 15/120), ST354 (9.2%; 11/120) and ST648 (8.3%; 10/120), which accounted for 45.0% (54/120) of all strains. Including the pandemic ST410 lineage, more than half of all strains (50.8%; 61/120) belonged to one of the five most prevalent STs in this study (**Figure 1**). These sequence types belonged to phylogroups B2 (ST131), D (ST405), and F (ST354, ST648) as well as phylogroup C (ST410), thus underlining the heterogeneity of phylogenetic backgrounds among ESBL-producing *E. coli*.

Phylogenetic Relationships

To elucidate phylogenetic relationships, we constructed a tree in an alignment-free manner for all investigated genomes (**Supplementary Figure 1**). Additionally, we inferred phylogenies, which are based on SNPs in the core genome of strains belonging to the five predominant STs of this study to assess potential transmission scenarios (**Figure 2**). For comparative reasons, we included 13 genomes of ESBL-producing *E. coli* isolated from houseflies (Heiden et al., 2020b) originating from the same Rwandan hospital.

The phylogenetic analysis (**Supplementary Figure 1**) shows that the *E. coli* strains were distributed among six distinct phylogroups and grouped into several clades according to their sequence type. Within these ST-associated phylogenies, several sub-clades were defined with genomes interspersed in patients, caregivers, related community members as well as animals and flies, which suggests common phylogenetic backgrounds (Clermont et al., 2011) potentially based on interspecies transmission. For example, PBIO458 (study-ID 60) and PBIO459 [(study-ID 133)—both ST38 isolated from two animals—clustered with four different strains from patients, family members and neighbors, PBIO272 (patient admission, study-ID 60), PBIO451 (neighbor, study-ID 60), PBIO455 (family member, study-ID 60), and PBIO467 (patient follow-up, study-ID 60)]. These findings are corroborated by results of two of our previous studies, where we demonstrated the likely transmission of ESBL-producing *E. coli* ST38 among humans and animals (Guenther et al., 2017; Schaufler et al., 2018). Note, however, that in this current study, only one representative of ST38 [PBIO302 (caregiver admission, study-ID 131)] carried a chromosomally encoded $bla_{CTX-M-15}$ gene and the before mentioned strains carried plasmid-encoded ESBLs (**Supplementary Figure 1**), which is contrary to our previous findings. We then compared the $bla_{CTX-M-15}$ gene-carrying chromosomal contig of PBIO302 to two of the plasmid-encoded $bla_{CTX-M-15}$ sequences of ST38 (PBIO272 and PBIO459; **Supplementary Figure 2**). The chromosomal sections of PBIO302, PBIO272, and PBIO459 were highly similar, except the chromosomal insertion of $bla_{CTX-M-15}$ in PBIO302. This resistance gene was flanked by transposable elements, as described below.

In addition, strains with the numbers PBIO1939, PBIO1942, PBIO1945, PBIO1946, and PBIO1947 from houseflies were in the same sub-clade as strains isolated from different human sources indicating the potential role of living vectors in the spread of pathogenic bacteria.

For the phylogenetic trees of the five predominant STs of this study (**Figure 2**) it is interesting to notice that some genomes stemming from different sources were more closely related than genomes from the same source. For example, PBIO283 [(study-ID 92) **Figure 2A**, ST131, sub-clade 1], which originates from a caregiver at admission differed in 0.2 ± 0.0003 SNPs/Mbp with strains isolated from the related patient at admission [PBIO286 (study-ID 92)] as well as discharge [PBIO285 (study-ID 92)] and unrelated patients at discharge [PBIO293 (study-ID 114) and PBIO296 (study-ID 117)]. Moreover, PBIO440 [(study-ID 133) **Figure 2A**, ST131, sub-clade 2], isolated from a community member, only varied in 0.2 SNPs/Mbp compared to a follow-up strain of an already discharged patient [PBIO405 (study-ID

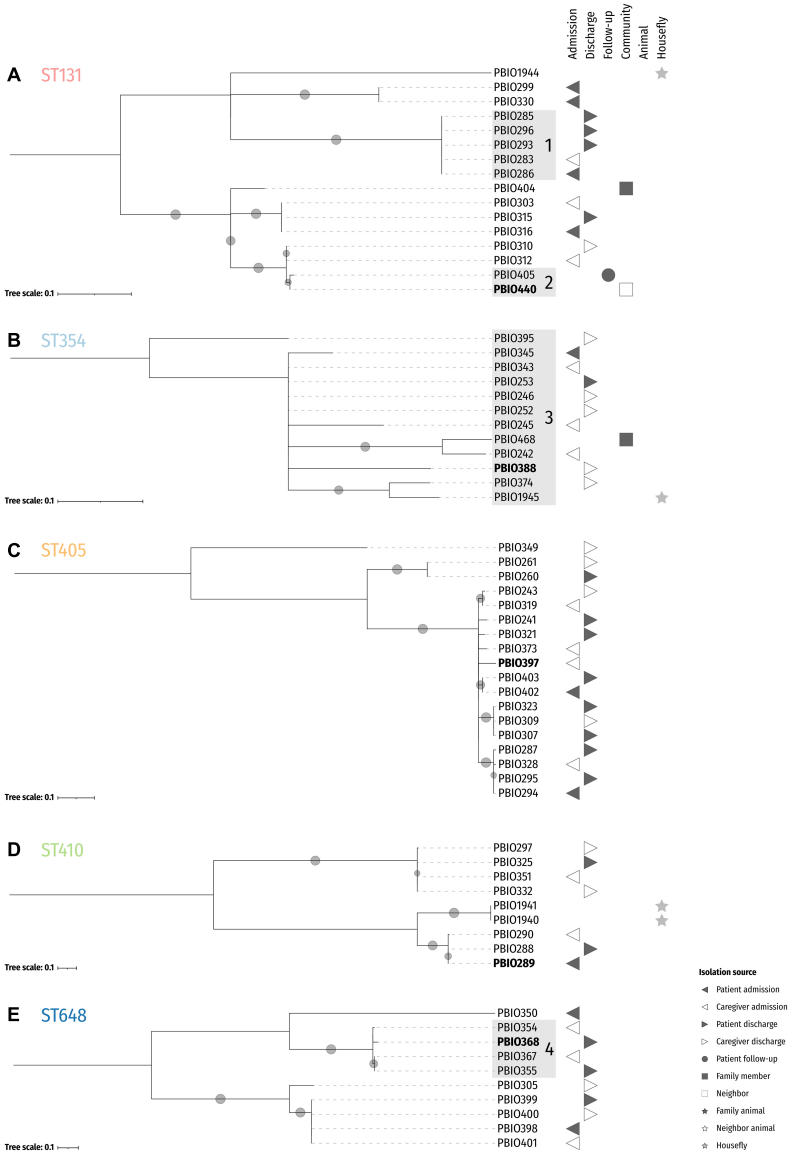

FIGURE 2 | Midpoint-rooted maximum likelihood core SNP phylogenies of the five dominating sequence types. Reference isolates for ST131 (PBIO440; **A**), ST354 (PBIO388; **B**), ST405 (PBIO397; **C**), ST410 (PBIO289; **D**), and ST648 (PBIO368; **E**) are highlighted in bold. The trees are based on alignments with 208 **(A)**, 20 **(B)**, 177 **(C)**, 154 **(D)**, and 135 **(E)** sites. Sub-clades are highlighted in gray. Circles at branches display bootstrap proportions ≥50% (1,000 replicates). Symbols besides the tree depict isolation sources as given in the legend.

434)]. Notably, all strains belonging to ST354 (**Figure 2B**, sub-clade 3) only differed in 1.0 ± 0.4 SNPs/Mbp including one strain carried by a housefly (PBIO1945). Also interesting was the difference between PBIO368 [(study-ID 335) **Figure 2E**, ST648, sub-clade 4], originating from a patient at discharge, and strains of distinct sources [PBIO354 (caregiver admission, study-ID 265), PBIO355 (patient discharge, study-ID 265), and PBIO367 (caregiver admission, study-ID 288)], differing in 0.9 ± 0.1 SNPs/Mbp. These numbers of SNPs were up to 10-fold lower than described for clonal EHEC strains during an outbreak in Germany (1.8 SNPs/Mbp) (Grad et al., 2012; Been et al., 2014), suggesting the circulation of only a handful of sequence types in this African setting, which interestingly happen to mostly be international high-risk clonal lineages. In addition, some strains from identical STs were carried by both flies and humans, for example PBIO1945 and PBIO374 (caregiver discharge, study-ID

401; **Figure 2B**), again indicating the role of flies in the spread of antibiotic-resistant pathogens.

Antimicrobial Resistance Determinants

The predominant ESBL-gene was $bla_{CTX-M-15}$ (87.5%; 105/120). Furthermore, 76 strains (63.3%; 76/120) carried bla_{OXA-1} and eight (6.7%; 8/120) $bla_{CTX-M-27}$. Previous studies reported the co-occurrence of $bla_{CTX-M-15}$ and bla_{OXA-1}, whereas other members of the CTX-M family (e.g., $bla_{CTX-M-27}$) and bla_{OXA-1} appear to be mutually exclusive (Schaufler et al., 2016b; Livermore et al., 2019; Bodendoerfer et al., 2020). Here, almost all strains carrying the bla_{OXA-1} gene (98.7%; 75/76) carried the $bla_{CTX-M-15}$ gene in addition and none showed the combination of bla_{OXA-1} and another CTX-M gene. The co-occurence of distinct CTX-M genes was not detected. For the majority of bla_{CTX-M}-positive strains (71.1%; 81/114), the corresponding gene was encoded on a plasmid (**Supplementary Figure 1**). Interestingly, all strains of phylogroup F (20.0%; 24/120) were carriers of $bla_{CTX-M-15}$ genes, of which 87.5% (21/24) were located on the chromosome (all strains belonging to ST648 and ST354). These chromosomally encoded genes were flanked by an insertion sequence IS*Ecp1* upstream and a Tn*2* downstream. Also note that the strains PBIO242 and PBIO245 (ST354) showed two of these motifs consecutively. The $bla_{CTX-M-15}$ gene of the ST410 strains PBIO288, PBIO289, and PBIO290 were found in proximity to an upstream-located IS*Ecp1* only. Previous studies have already demonstrated the diversity and global distribution of these complex transposable units in enterobacteria (Poirel et al., 2005; Lartigue et al., 2006; Decano and Downing, 2019; Ludden et al., 2020; Yoon et al., 2020).

Genes encoding for carbapenem-hydrolyzing enzymes, like bla_{OXA-48} or bla_{NDM-1}, were not present.

A growing body of ESBL-producers is MDR (Cantón and Coque, 2006; Hidron et al., 2008; Pitout, 2012; Beceiro et al., 2013). In our study, all investigated strains (100.0%; 120/120) were carriers of genes conferring resistance to three or more different classes of antimicrobial agents, thus exhibiting a MDR genotype (Magiorakos et al., 2012). In total, 120 strains (100.0%; 120/120) carried resistance genes to tetracyclines (*tet*), followed by genes encoding for aminoglycoside (*aac, aad*, and *aph* [98.3%; 118/120]), sulfonamide [*sul* (90.0%; 108/120)], trimethoprim [*dfr* (89.2%; 107/120)], chloramphenicol [*cat* (72.5%; 87/120)], and fluoroquinolone [*aac(6′)-Ib-cr* and *qnr* (71.7%; 86/120)] resistances. Two strains (1.7%; 2/120) belonging to ST181 and ST540 were carriers of *mcr-9* but showed phenotypic susceptibility to colistin, which is a last-resort antibiotic (MIC of both strains: 0.5 μg/mL). Interestingly, previous studies suggest that this latest member of the *mcr* gene family does not always confer phenotypic resistance to colistin in clinical isolates, although overexpression in *E. coli* TOP10 cells leads to increased MICs (Carroll et al., 2019; Kieffer et al., 2019; Tyson et al., 2020).

In addition to the resistances described, genes encoding for efflux pumps were found frequently, with *mdfA* in all (120/120) and *acrB* in 85.8% (103/120) of all genomes.

Virulence-Associated Genes

As previously demonstrated by us and others, the combination of MDR and high-level bacterial virulence seems to be a hallmark of pandemic high-risk clonal lineages (Hussain et al., 2012; Ewers et al., 2014; Shaik et al., 2017; Schaufler et al., 2019). To investigate the strains' genetic virulome, we analyzed the genomes of the predominant ST131 (n = 15), ST648 (n = 10), ST354 (n = 11), ST405 (n = 18), and ST410 (n = 7) for these features (**Figure 3**).

The strains belonging to the five predominant STs carried several VAGs, mainly associated with adherence, antiphagocytosis, biofilm formation, invasion, iron uptake and bacterial secretion. The ability to attach to surfaces/cells and form biofilms is a common strategy used by bacterial populations to resist antibiotic treatment and host defense mechanisms as well as cause infection (Moser et al., 2017; Amanatidou et al., 2019). In particular, genes for the P fimbriae adhesion cluster [*pap* operon (70.5%; 43/61)], Dr family of adhesins (4.9%; 3/61) and type I fimbriae [*fim* (100%; 61/61)], which are necessary for uroepithelia cell adhesion and invasion, and, thus, for causing urinary tract infection (Mulvey, 2002), were frequently found. Notably, strains belonging to ST354 and ST410 showed a lack of *pap* genes, which is consistent with previous findings (Vangchhia et al., 2016; Zogg et al., 2018; Schaufler et al., 2019). Furthermore, we detected several members of the *csg* gene family in all genomes (100%; 120/120). These genes encode curli fibers, which are essential components of bacterial biofilms (Hammar et al., 1995; Evans and Chapman, 2014).

The ability to acquire intracellular heme and hemolysin, which is based on the expression of iron uptake-associated genes [e.g., Chu heme uptake system (88.5%; 54/61), yersiniabactin (86.9%; 53/61), and aerobactin (65.6%; 40/61)], is an effective strategy for iron utilization during infection (Fischbach et al., 2006) and is another important virulence-associated feature in the repertoire of these bacteria.

Mobile Genetic Elements

We next investigated the occurrence and circulation/transfer of ESBL-plasmids among strains and thus their contribution to the spread of antibiotic resistance in the Rwandan setting.

In total, 107 strains (89.5%; 107/120) carried plasmids with incompatibly (Inc) group FIB, followed by IncFIA (75.8%; 91/120) and IncFII (70.8%; 85/120). In particular IncF plasmids are frequently associated with genes encoding ESBL-genes, other resistances as well as virulence features important for iron acquisition (Han et al., 2012), serum resistance (Ranjan et al., 2018), and biofilm formation (Schaufler et al., 2016a).

To better assess similar plasmid backgrounds, we compared the plasmid sequences of all strains that carried a plasmid-borne $bla_{CTX-M-15}$ gene against the plasmid sequence of PBIO241 (ST405; patient discharge; study-ID 159; **Figure 4**) as a representative for the most prevalent ST. Keep in mind, however, that the selection of the reference biases the visual representation when a large number of plasmid sequences is absent in the query sequences. On the other hand, plasmid sequences, not present in the reference but in queries, are missing in this approach. The plasmid backbones of other ST405

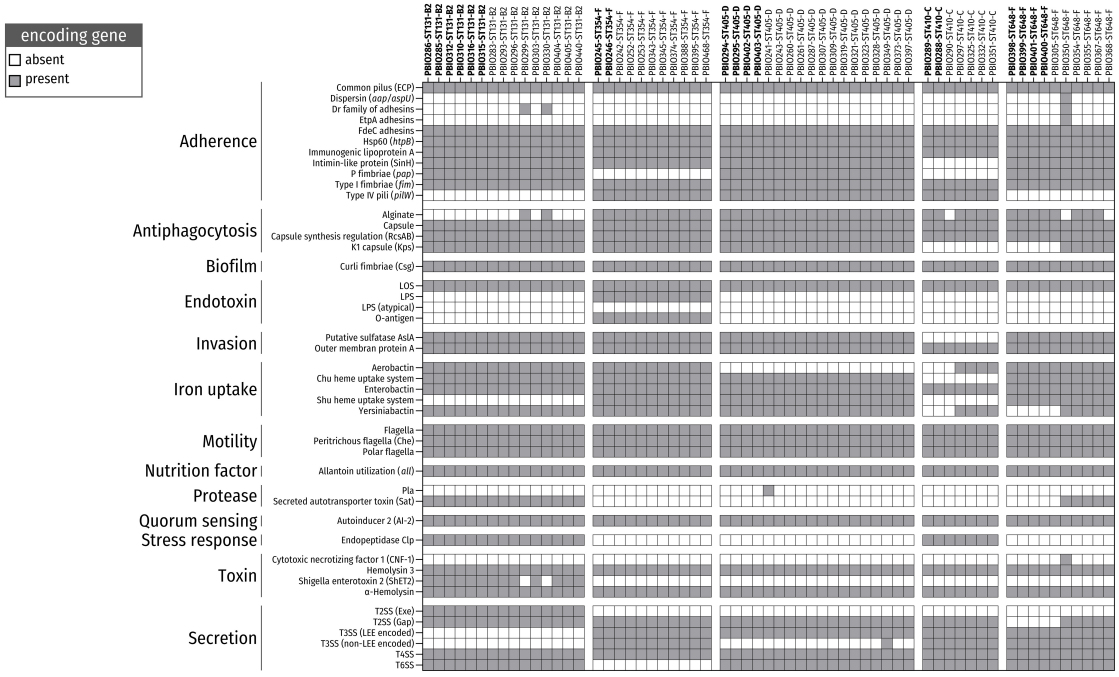

FIGURE 3 | Presence/absence of virulence factors (VAGs) among ESBL-producing *E. coli* belonging to the five most prevalent sequence types (STs). Color-filled boxes show the presence of genes (coverage and identity ≥ 65%) encoding for different VAGs.

strains were highly similar to the reference. In contrast, plasmids from strains of other pandemic STs (ST131 and ST410) only showed few similarities when compared to the reference plasmid underlining their genetic diversity. Interestingly, in addition, the plasmid sequences of four ST167 strains [PBIO276 (patient discharge, study-ID 78), PBIO277 (patient admission, study-ID 78), PBIO301 (caregiver discharge, study-ID 131), and PBIO306 (patient discharge, study-ID 131)] were highly similar to the reference. Five other strains [PBIO256 (ST12, patient admission, study-ID 40), PBIO257 (ST12, patient discharge, study-ID 40), PBIO258 (ST1421, patient admission, study-ID 40), PBIO1948 (ST5474, housefly), and PBIO1949 (ST5474, housefly)] showed identical plasmid sequences in parts of the BRIG visualization that were absent in most of the other sequences.

We also compared some of our plasmid sequences of representative strains (criteria: top BLAST-hits) against a publicly available reference plasmid pIV_IncHI2_CTX_M_15 (Marchetti et al., 2020; **Supplementary Figure 3**). This $bla_{\mathrm{CTX-M-15}}$-containing plasmid has been originally obtained from an *E. coli* ST58 strain causing a deadly puppy infection in Italy in 2019. Interestingly, the plasmid backbone of PBIO251 (ST155, patient admission, study-ID 153) was nearly identical when compared to this external Italian plasmid. The plasmids of PBIO275 (ST540, caregiver admission, study-ID 78), PBIO385 (ST3494, patient discharge, study-ID 434), and PBIO1943 (ST5474, housefly) also showed high similarities to the reference and thus among each other.

The presence of similar resistance plasmids in strains of distinct sequence types and sample groups indicates the potential transmission of mobile genetic elements and the growing prevalence of successful plasmid families (Carattoli, 2011).

DISCUSSION

In contrast to Europe, the United States of America and Australia, only little information is available concerning the exact characteristics and distribution of ESBL-producing *E. coli* in Africa. This study reveals the broad occurrence and potential circulation of several international high-risk clonal lineages in a hospital and associated locations in Rwanda, following up on a publication by Kurz et al. (2017). Despite the potential bias due to the analysis of both admission and discharge strains of the same patient and/or caregiver, it is remarkable that our sample set was dominated by ST131, ST648, and ST410, which have been frequently reported from humans, animals and the environment (Nicolas-Chanoine et al., 2008; Ewers et al., 2010, 2014; Hussain et al., 2012; Schaufler et al., 2016b, 2019) and which are "classic" pandemic, high-risk clonal lineages. In addition to these, we found ST354 to be the third most detected sequence type in this study. Its emergence has been previously described globally except for the African continent (Manges et al., 2019). This locally restricted accumulation of one single ST in combination with a small number of other STs indicates re-entering and circulation of

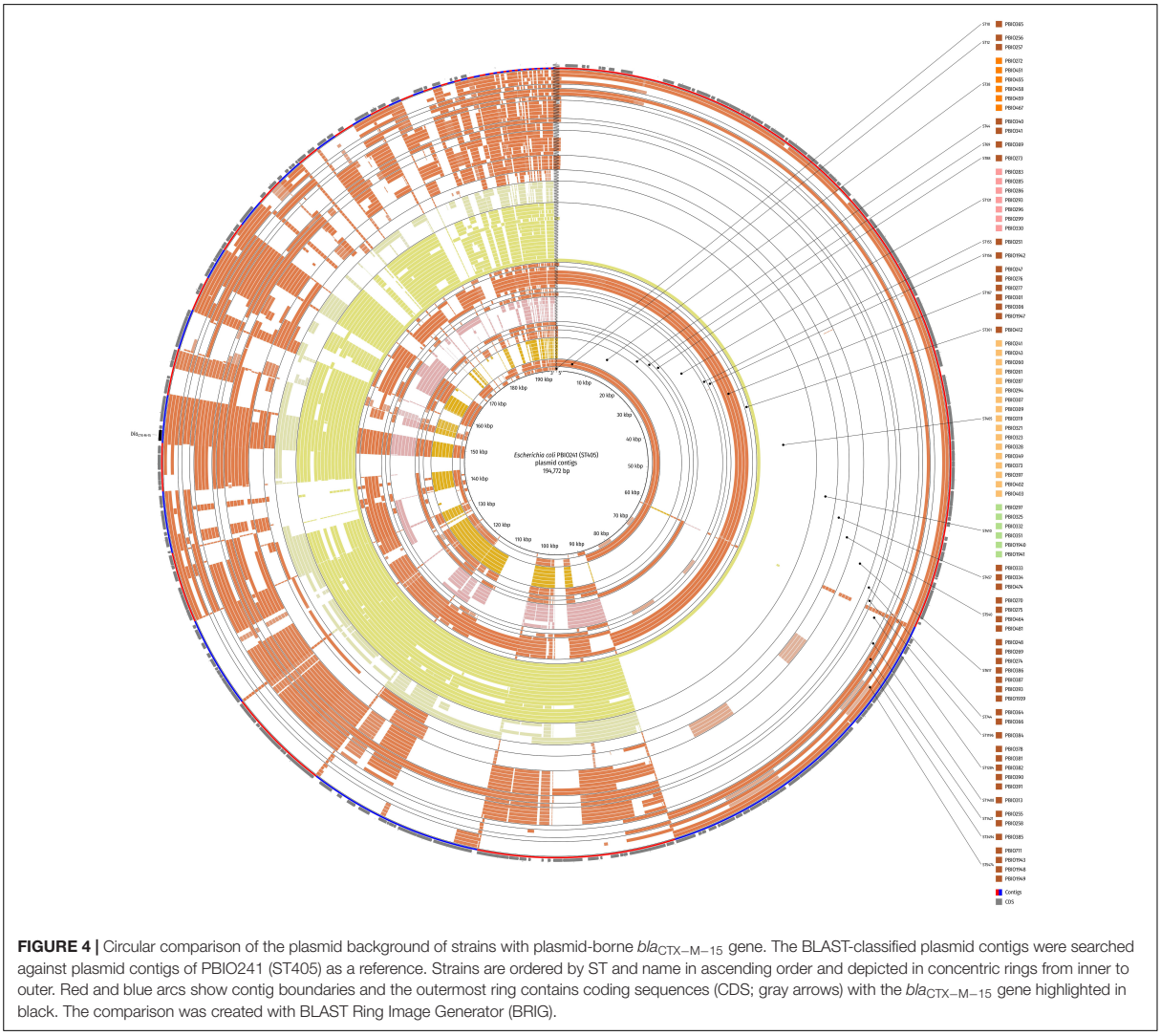

FIGURE 4 | Circular comparison of the plasmid background of strains with plasmid-borne $bla_{CTX-M-15}$ gene. The BLAST-classified plasmid contigs were searched against plasmid contigs of PBIO241 (ST405) as a reference. Strains are ordered by ST and name in ascending order and depicted in concentric rings from inner to outer. Red and blue arcs show contig boundaries and the outermost ring contains coding sequences (CDS; gray arrows) with the $bla_{CTX-M-15}$ gene highlighted in black. The comparison was created with BLAST Ring Image Generator (BRIG).

dominant bacterial lineages in the Rwandan hospital and among family members/neighbors and animals.

Interestingly, within the ST-associated clades, some genomes without genetic differences were interspersed in humans (hospitalized patients and caregivers as well as community members) and animals. This putative lack of host adaptation and the close phylogenetic relationships indicate the colonization and rapid transmission of several clones within the community and the potential transmission into the clinical setting and vice versa, underlined by the high acquisition rates of ESBL-producing *E. coli* during hospitalization as described previously (Kurz et al., 2017).

The resistance genes found in this study confer resistances to antibiotics frequently used in veterinary medicine and/or in sub-therapeutic doses as food supplements and growth promotors in Africa (Eagar et al., 2012; Adesokan et al., 2015; Mainda et al., 2015; Manishimwe et al., 2017). When also considering the zoonotic character of ESBL-producing *E. coli*, it is not surprising that we found clonal strains with similar patterns of resistance features in the different sample groups. Transmission likely occurred among patients and caregivers/family members and was also influenced by livestock animals due to close human-animal contact (Klous et al., 2016).

Two strains of this study carried the *mcr-9* gene but were phenotypically susceptible to colistin. This phenomenon was first reported in 2019 (Carroll et al., 2019; Kieffer et al., 2019). Due to the structural heterogeneity compared to other *mcr* genes (65% amino acid identity with the most closely related *mcr-3* gene) and the weak inactivation of colistin, the clinical importance of mcr-9 is unknown (Tyson et al., 2020).

Notably, some of the strains showed extensive, chromosomally encoded virulence-associated features. The CNF-1-encoding

gene (*cnf1*) detected in strain PBIO350 (ST648), for example, is associated with causing neonatal meningitis (Khan et al., 2002). In addition to the major virulence factors of meningitis-associated and uropathogenic *E. coli* (like P fimbriae adhesion cluster, K1 capsule, heme utilization systems, and the secreted autotransporter toxin), the strains showed VAGs typical for InPEC (especially the various bacterial secretion systems) underlining the clinical relevance of these pathogens.

Finally, we demonstrate that similar plasmid sequences were present in strains from different sample groups, thus likely indicating mobile genetic element transmission, and underlining the importance of plasmid-driven spread of antimicrobial resistance independent of the host's phylogenetic background (Schaufler et al., 2016a; Ranjan et al., 2018). Interesting in addition to similarities among strains from the Rwandan setting is in particular the close relationship to an external plasmid, which has been obtained only recently (Marchetti et al., 2020). This highlights the sometimes global spread of such mobile genetic elements and their bacterial hosts.

CONCLUSION

In this study, we investigated and identified the presence of clinically relevant ESBL-producing *E. coli* that circulate among patients, caregivers, the community and animals in a Rwandan setting. The findings contribute to the understanding of the global dissemination of bacterial high-risk clonal lineages, their virulence features as well as plasmid transmissions. They also underline the potential role of houseflies in this harmful dynamic.

DATA AVAILABILITY

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB42795 (https://www.ebi.ac.uk/ena/browser/view/PRJEB42795).

AUTHOR CONTRIBUTIONS

KS and EE designed the study. EE and JM performed the laboratory and phenotypic experiments. SH, SS, and KK performed the bioinformatics analyses. KS, EE, SH, KK, CB, JN, AS, JG, MK, FM, and SS analyzed the data. KS, EE, and SH wrote the manuscript and prepared the tables and figures. All authors read and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.662575/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.3 Publication 3

The study "**Highly virulent and multidrug-resistant *Escherichia coli* sequence type 58 from a sausage in Germany**" combined functional genomics with a comprehensive set of *in vitro* and *in vivo* phenotyping of the food-borne *E. coli* ST58 strain PBIO3502 isolated from a fresh pork sausage bought in Germany.

PBIO3502 was resistant to several antibiotics as well as heavy metals and possessed a variety of virulence-associated genes mainly associated with adherence, biofilm formation, invasion, iron uptake, and motility. *In vitro* experiments showed that PBIO3502 formed curli fibers and cellulose and was thus positive for biofilm formation. In addition, it secreted a large amount of siderophores and demonstrated resilience against complement-containing human serum. An *in vivo* infection model with *G. mellonella* larvae suggested high mortality, comparable to a hvKp strain, while phylogenetic analysis placed it in a group consisting mainly of strains from domestic and wild animals from different European regions. However, phylogenetically related ST58 genomes of human and animal origin in other sub-clades indicated the zoonotic character of the ST58 lineage [120].

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Contributions

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Stefan E. Heiden	data curation, formal analysis, software, visualization
Madeleine Paditz	formal analysis, investigation
Veronika Balau	formal analysis, investigation
Christiane Huxdorff	formal analysis, resources
Dirk Zimmermann	formal analysis, resources
Timo Homeier-Bachmann	formal analysis, resources, funding acquisition
Katharina Schaufler	conceptualization, project administration, supervision, funding acquisition, writing – review and editing

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MDPI

Communication

Highly Virulent and Multidrug-Resistant *Escherichia coli* Sequence Type 58 from a Sausage in Germany

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Abstract: Studies have previously described the occurrence of multidrug-resistant (MDR) *Escherichia coli* in human and veterinary medical settings, livestock, and, to a lesser extent, in the environment and food. While they mostly analyzed foodborne *E. coli* regarding phenotypic and sometimes genotypic antibiotic resistance and basic phylogenetic classification, we have limited understanding of the in vitro and in vivo virulence characteristics and global phylogenetic contexts of these bacteria. Here, we investigated in-depth an *E. coli* strain (PBIO3502) isolated from a pork sausage in Germany in 2021. Whole-genome sequence analysis revealed sequence type (ST)58, which has an internationally emerging high-risk clonal lineage. In addition to its MDR phenotype that mostly matched the genotype, PBIO3502 demonstrated pronounced virulence features, including in vitro biofilm formation, siderophore secretion, serum resilience, and in vivo mortality in *Galleria mellonella* larvae. Along with the genomic analysis indicating close phylogenetic relatedness of our strain with publicly available, clinically relevant representatives of the same ST, these results suggest the zoonotic and pathogenic character of PBIO3502 with the potential to cause infection in humans and animals. Additionally, our study highlights the necessity of the One Health approach while integrating human, animal, and environmental health, as well as the role of meat products and food chains in the putative transmission of MDR pathogens.

Keywords: antimicrobial resistance; CTX-M-1; *Enterobacterales*; *Escherichia coli*; food safety; IncI1; One Health

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1. Introduction

Multidrug-resistant (MDR) *Enterobacterales*, such as *Escherichia coli*, which are non-susceptible to more than three classes of antibiotics [1], have been on the rise for years. They are not only found in human and veterinary clinics but also in livestock and related animal products. Therefore, they are an important component of the One Health approach [2,3], which aims at increasing and/or preserving the well-being of human, animal, and environmental health. While most One Health reports in this research area focus on the distribution of antibiotic resistance and phylogenetic backgrounds, such as sequence types (STs), little is known about phenotypic virulence traits in vitro and in vivo [4]. The latter is particularly important when considering the zoonotic nature of MDR *E. coli* from animal sources, which have the potential to infect humans, and vice versa. These pathogenic

E. coli are divided into extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic representatives (InPEC) [5], with the latter exhibiting obligate pathogenicity, and foodborne isolates potentially causing outbreaks, such as in Germany in 2011 [6,7]. In contrast, ExPEC strains usually colonize the intestinal tract of humans and animals asymptomatically but frequently cause infection outside the gut. ExPEC possess several virulence factors (VFs) that enable adhesion, biofilm formation, resilience and, thus, infection in human and/or animal hosts, and are critical for bacterial pathogenicity. However, the exact contribution of foodborne MDR ExPEC to human and animal infection and colonization has not yet been established beyond doubt, as the differentiation between commensal *E. coli* and facultative ExPEC strains remains difficult when performing either geno- or phenotypic characterization alone [8].

Here, we report an extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolated from a pork sausage in Germany that we investigated in-depth by whole-genome sequencing (WGS) and functional analyses. The aim of this study was to (i) identify the phylogenetic background of the food-derived strain and explore its phylogenetic relationship to other genomes of the same sequence type, and (ii) examine the repertoire of genotypic and phenotypic resistance- and virulence-associated features combining bioinformatics with multiple in vitro and in vivo assays.

2. Results

2.1. Genomic Characterization

As part of a small screening study for which we analyzed various meat products (including fresh pork sausage) from several grocery stores in Germany in May 2021, we isolated the ESBL-producing *E. coli* strain PBIO3502, which was first verified by MALDI-TOF MS and subsequently analyzed in detail by WGS. WGS revealed that PBIO3502 belonged to ST58 (serotype O65:H16) and, thus, phylogroup B1, which includes mostly commensal but also some ExPEC and InPEC strains [9]. PBIO3502 carried plasmids with the incompatibly groups (Inc)FIA and IncI1, as well as Col plasmids. Plasmid multilocus sequence typing [10] revealed that the IncI1 plasmid was ST3 (clonal complex 3).

The presence of resistance genes was investigated using AMRFinderPlus [11] and revealed the presence of the ESBL gene $bla_{\text{CTX-M-1}}$ (Ambler class A) and a BlaEC family class C β-lactamase bla_{EC} (98.67% identity to EC-15) in addition to genes conferring resistance to macrolides (*mdf(A)*, *mef(C)*, and *mph(G)*), streptomycin (*aadA5*), sulfonamides (*sul2*), and diaminopyrimidines (*dfrA17*). Our phenotypic analysis of antibiotic resistance was mostly consistent with the genotype (Table 1) and showed that PBIO3502 had an MDR phenotype [1]. Additionally, we noticed the presence of heavy metal resistance-associated genes (*terWZABCDE*) that enable resistance to tellurite. Usually, *E. coli* strains exhibit minimum inhibitory concentrations of tellurite of 1 μg mL^{-1} [12], but PBIO3502 showed a 64-fold increased tolerance.

Among the nearly 300 genes associated with *E. coli* virulence provided by the virulence factor database (VFDB) [13], PBIO3502 harbored several gene clusters mainly associated with adherence (CFA/I fimbriae, *cfaABCD/E*; *E. coli* common pilus, *ecpABCDER*; factor adherence *E. coli*, *fdeC*; Hsp60, *htpB*; immunogenic lipoprotein A, *ilpA*; type I fimbriae, *fimABCDEFGHIZ*; type IV pili, *vfr*), biofilm formation (alginate, *algU*; autoinducer-2, *luxS*; curli fibers, *csgABCDEFG*), invasion (*ibeBC*, *ompA*, *traJ*), iron uptake (aerobactin, *iucABCD*, *iutA*; enterobactin, *entABCDEFS*, *fepABCDEG*, *fes*; salmochelin, *iroB*), and motility (peritrichous flagella, *cheBRWYZ*, *flgBCDFGHI*, *flhABCD*, *fliAGIMPQR*, *motA*; polar flagella, *flmH*, *nueA*). Genes encoding Shiga toxin were not present.

Table 1. Phenotypic and genotypic resistance profile of PBIO3502.

Antimicrobial Category	Antimicrobial Agent	MIC [a] [μg mL^{-1}]	S/R [b]	Genotype [d]
Aminopenicillin + β-lactamase inhibitor	Ampicillin/sulbactam	≥32/16	R	bla_{EC}
Ureidopenicillin + β-lactamase inhibitor	Piperacillin/tazobactam	≥128/4	R	bla_{EC}
Third generation cephalosporins	Cefotaxime Ceftazidime	≥64 ≥64	R R	$bla_{CTX\text{-}M\text{-}1}$
Carbapenems	Meropenem	≤0.25	S	
Aminoglycosides	Gentamicin Streptomycin	≤0.5 64	S *NA*	*aadA5*
Fluoroquinolones	Ciprofloxacin	≤0.25	S	
Tetracyclines	Tetracycline	≤0.5	S [c]	
Folate pathway inhibitors	Trimethoprim/sulfamethoxazole	≥16/304	R	*dfrA17*, *sul2*
Phosphonic acids	Fosfomycin	≤16	S	

[a] MIC, minimum inhibitory concentration; [b] Interpretive categories according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0. 2022). S, susceptible; R, resistant; *NA*, not applicable. [c] Result was interpreted according to the published breakpoints of the CLSI (Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 32nd edition. 2022). [d] Prediction based on alignment of sequences from AMRFinderPlus database (threshold for coverage and identity, ≥80%).

2.2. Phenotypic Assessment of Virulence-Associated Traits

Given the potential zoonotic background of PBIO3502 and this large genomic set of VFs, we further characterized PBIO3502 in several phenotypic virulence-associated experiments (Figure 1).

Some bacterial populations are able to protect themselves from external stressors (e.g., UV radiation and antibiotic therapy) by forming a biofilm [14]. In addition, there is evidence that biofilm formation is a critical factor in infection development [15]. Therefore, we investigated the ability of PBIO3502 to form biofilms and analyzed whether the strain formed curli fibers (bacterial amyloid fibers) and/or cellulose (polysaccharide structures), which are important biofilm- and, thus, virulence-associated features [16]. When compared to the strong biofilm producer *E. coli* W3110 (specific biofilm formation (SBF) median, 1.96), PBIO3502 showed high adhesiveness to the plastic surface of a 96-well plate (SBF median, 2.86; Figure 1a), which likely promotes biofilm formation on catheters [17], for example. In addition, PBIO3502 showed both curli and cellulose formation in the long-term colony. The dye was used to assess the morphology of long-term colony stains only the curli fibers, but not the cellulose structures [18]. The latter are interpreted visually based on the structured surface (Figure 1b). Calcofluor, which has a high affinity for cellulose [19], was used to obtain a (semi-) quantitative result on the cellulose formation of PBIO3502. Our strain showed pronounced cellulose formation indicated by high fluorescence intensities (Figure 1c).

Iron is an important element for pathogenic bacteria, especially during infection, but also for general growth and fitness [20]. Since bacteria cannot produce iron themselves, they have to acquire it from their surroundings. Therefore, pathogens produce low-molecular iron-binding molecules (so-called siderophores) that chelate free extracellular iron(III) and are subsequently internalized by membrane receptors. We examined whether and to what extent PBIO3502 produced siderophores, and as shown in Figure 1d, PBIO3502 secreted large amounts of siderophores (mean, 87.25%). The extent of siderophores was 11.5-fold higher when compared to the negative control W3110.

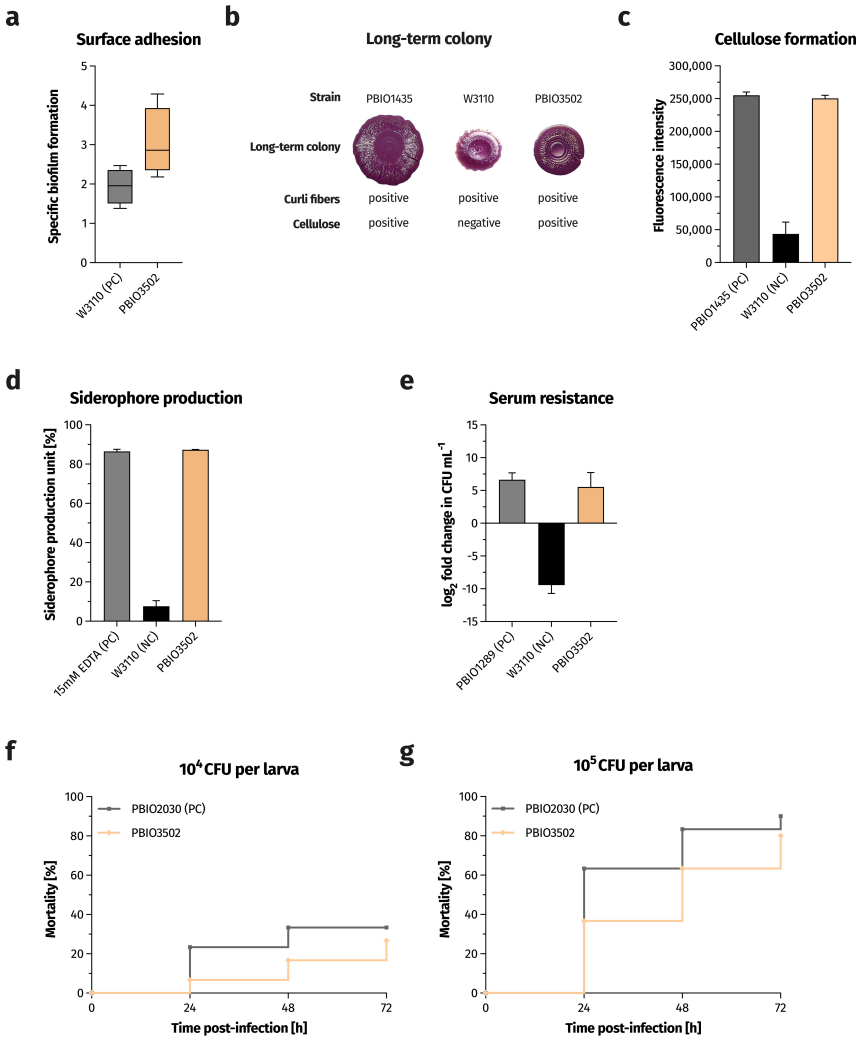

Figure 1. PBIO3205 exhibits a highly virulent phenotype. (**a**) Biofilm formation on polystyrene surfaces as determined by crystal violet staining (n = 4). Results are expressed as growth-adjusted specific biofilm formation. The line within the box marks the median value, while the boxplot represents the 25th to 75th percentile of the data set. The whiskers mark the minimum and maximum values, respectively. (**b**) Morphology of long-term colonies examining the expression of the biofilm-associated extracellular matrix components curli fibers and cellulose. Congo red was used to specifically stain curli fibers, and cellulose was visually interpreted based on the textured surface. PBIO1435 and W3110 were used as references. (**c**) Cellulose formation was examined by staining with Calcofluor and measuring the fluorescence intensity of bound Calcofluor (n = 3). Results are given as mean values of fluorescence intensity and standard errors. (**d**) The extent of secreted siderophores is expressed as the mean of the percent unit of siderophore production and standard error (n = 3–5). (**e**) Survival in 50% human serum (n = 3–5). Results are given as means and standard errors of $\log_2$ fold change in CFU mL^{-1} after 4 h of incubation in the presence of human serum. (**f**,**g**) Kaplan–Meier plot of mortality in the *Galleria mellonella* larvae infection model (n = 3). Results are expressed as mean percent mortality after injection of 10^4 CFU per larva (**g**) and 10^5 CFU per larva, respectively. NC, negative control. PC, positive control.

Highly virulent *E. coli* representatives are capable of causing bacteremia [21] and, therefore, must compete with the host defense system (e.g., the complement system). Gram-negative bacteria use several different mechanisms to deal with the immune response provided by various mechanisms, such as capsule expression [22], down-regulation of envelope stress response pathways [23], and outer membrane surface modifications [24]. Therefore, we challenged this foodborne strain by incubating it in 50% human serum for 4 h. PBIO3502 was not inhibited by the complement-containing serum and was able to double its cell number (Figure 1e). This result again highlights the highly virulent and clinically relevant nature of PBIO3502.

In addition, we performed an infection model using larvae of the greater wax moth *Galleria mellonella* to investigate the ability of PBIO3502 to cause in vivo mortality. We injected 10^4 or 10^5 colony forming units (CFU) per larva. When 10^4 CFU were injected, a mortality rate of 6.7% was obtained after 24 h (Figure 1f). This mortality rate increased to 26.7% 72 h after infection. The 10-fold increase in inoculation size resulted in an increase in the mortality rate to 36.7% at 24 h and 80.0% at 72 h of infection (Figure 1g). These results were comparable to the mortality rates of a hypervirulent *Klebsiella pneumoniae* ST420 strain PBIO2030 isolated from a wound [25]. In conclusion, the findings demonstrate the virulent potential of PBIO3502.

2.3. Phylogenetics of ST58

Finally, to classify our strain in a more global perspective, and again evaluate the zoonotic potential, we performed a phylogenetic comparison of an overall 159 closely related, publicly available ST58 genomes and PBIO3502 (Figure 2).

The phylogenetic tree shows that the genomes grouped into multiple clades with several subclades. Dispersed across the clades, genomes originated from humans, livestock, companion and wild animals, food, and the environment. Note that PBIO3502 clustered with three genomes from livestock (ERR3393207, ERR7420888) and environmental sources (SRR13848069), respectively. Interestingly, these closely related strains were sampled from different European locations in different years and were mainly associated with pigs. For example, ERR7420888 (designated MSG45-C09) was isolated from a healthy pig on a commercial pig farm in the Midlands of the United Kingdom in 2015 [26], while SRR13848069 (designated 20-MO00084-0) originated from scalding water from a pig slaughterhouse in Germany in 2020 [27]. All genomes assigned to the "PBIO3502 and ERR1055859 subclade" encoded aerobactin and tellurite resistance genes, a combination that was unique in the phylogenetic tree. Although PBIO3502 did not show immediate phylogenetic relatedness to human-derived strains and was in a clade primarily composed of strains from domestic and wild animals and the environment, our analysis demonstrates that phylogenetically related ST58 strains from humans were present in other clades. This again indicates the zoonotic character of the ST58 lineage and highlights the complex connections within the One Health continuum.

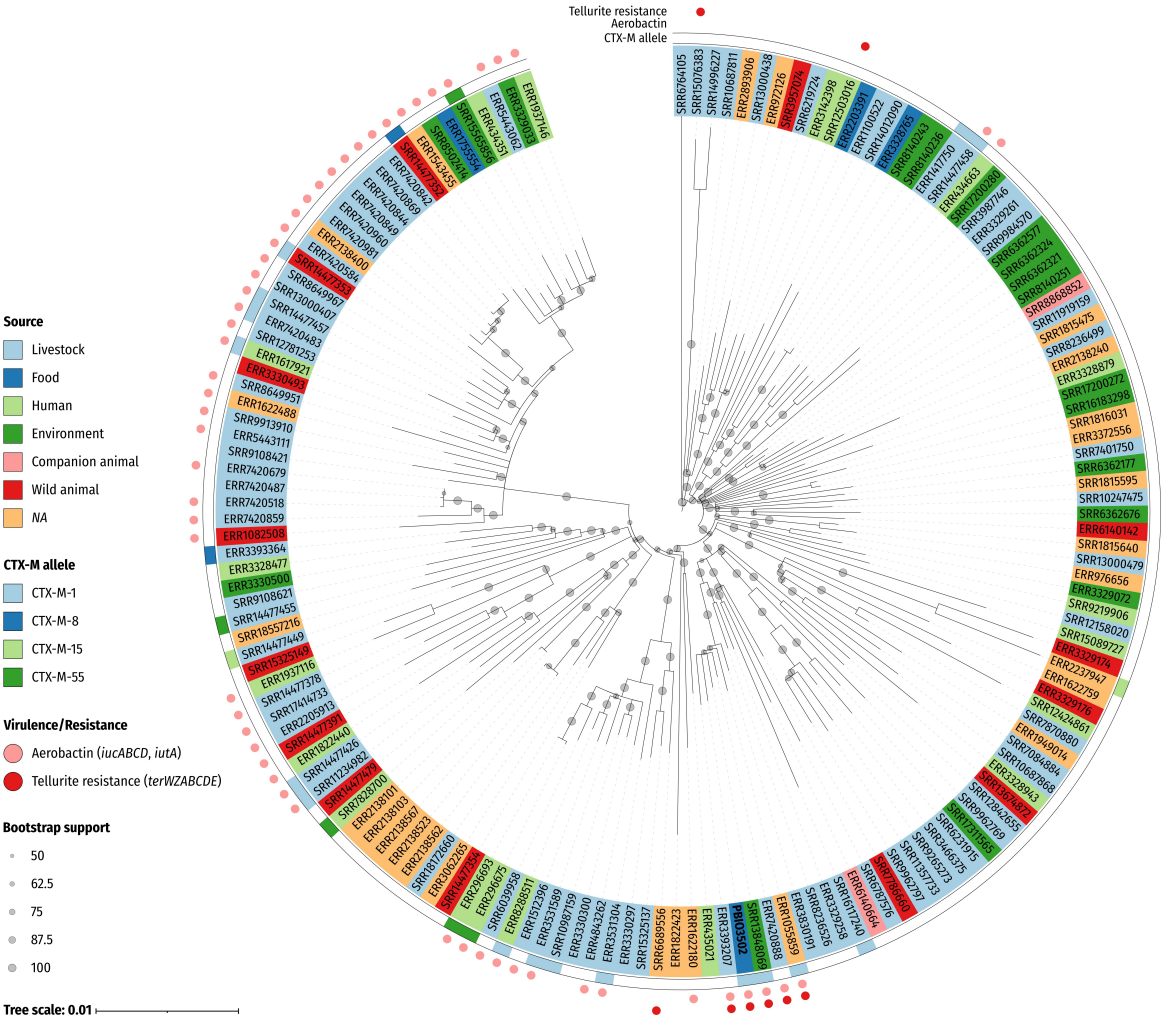

Figure 2. Phylogenetics reveals close relationship of PBIO3502 with publicly available genomes (n = 159). Included genomes were selected based on most shared k-mers using Mash. The phylogenetic tree was constructed using a maximum likelihood-based approach and is based on a core singe-nucleotide polymorphism alignment (4984 sites). The circular tree was midpoint-rooted and the circles on the branches indicate bootstrap support of ≥50% from 1000 replicates. The labels indicate the accession number (except PBIO3502) and are colored according to their source/host, as indicated in the legend. Annotations indicate (from inner to outer circle): encoded CTX-M (cefotaximase) allele, presence of genes encoding aerobactin and tellurite resistance. *NA*, not applicable (i.e., no metadata provided).

3. Discussion

A growing body of studies shows the global spread of MDR *E. coli* and underlines the clinical relevance of this pathogen, with nearly 60,000 deaths caused by antibiotic-resistant *E. coli* in 2019 alone [28]. In addition, MDR *E. coli* strains are commonly found in the environment, in animals (pets and livestock), and in meat products [2,29–34]. Due to increasing worldwide meat consumption and pressure on the prices of processed foods, slaughterhouses have to operate in an economically-oriented high throughput, potentially leading to the contamination of meat products and the water used with enteric pathogens of animal origin [35]. Therefore, it is not surprising that we and others have recently detected

MDR *E. coli* strains in the wastewater of German slaughterhouses that are even resistant to the last-resort drug colistin [27,36]. Moreover, contaminated meat products might foster the transmission of pathogens from animals to humans through the food chain, of which a confirmed MDR ExPEC outbreak in London (United Kingdom) in the mid-1980s [37–39] and the above-mentioned outbreak in Germany in 2011 are good examples. This underlines the interdependence of animal and human health and, thus, the need for the One Health approach. Although direct consumption of unprocessed fresh pork and poultry products is uncommon in Germany, putative foodborne ExPEC transmission involves the ingestion of inadequately cooked meat or cross-contamination in the kitchen during food handling, potentially, for example, resulting in wound infection [40,41].

The establishment and frequent application of next-generation sequencing technologies have demonstrated that the spread of MDR *E. coli* appears to be driven by a limited number of pandemic STs such as ST131, ST648, or ST410 [42–44]. These pathogenic clonal lineages successfully combine multidrug resistance and high virulence. However, other successful clonal ExPEC lineages, such as ST58 (which belongs to clonal complex 87 [45]), increasingly emerge as globally disseminated pathogens. PBIO3502 not only exhibited numerous virulence-associated characteristics, such as strong biofilm formation, extensive siderophore secretion, and serum resistance as well as in vivo mortality, but also demonstrated a MDR phenotype, supporting the above-mentioned previous findings. Additionally note that the phenotypic results were largely consistent with predictions from our genotypic analysis.

Unlike most pandemic ExPEC lineages, which are mostly associated with phylogroups B2 or D, ST58 belongs to phylogroup B1. Interestingly, this phylogroup contains only a small number of phylogroup-specific core genes compared to other phylogroups, indicating high diversity within this phylogroup [46]. Here, we describe a foodborne *E. coli* ST58 strain that exhibits pronounced in vitro and in vivo virulence properties required for pathogenicity and bacterial survival, indicating the great potential to actually cause infection in humans and animals upon successful transmission. While the direct transfer of *E. coli* ST58 strains via the food chain has not yet been confirmed beyond doubt [4,47], and is also not within the scope of this study, it has been previously suggested that ST58 shows high clonality among strains originating from humans, animals, and the environment [48–50]. Our phylogenetic analysis comparing global ST58 genomes supports the latter and reiterates the clinical and zoonotic relevance of our strain. However, we used a limited number of genomes in this study. Future investigations will need to address this further and provide a more comprehensive phylogenetic and epidemiologic picture of this (putatively) successful, emerging clonal lineage.

Many resistance genes, but especially ESBL enzymes, are often encoded on plasmids, which allows rapid resistance transfer among different bacteria and, thus, widespread dissemination [51]. Interestingly, PBIO3502 carried an IncI1 plasmid, which appears to be a common plasmid type of InPEC strains, but also, to a lesser extent, occurs in ExPEC [52]. Moreover, ST3 IncI1 plasmids are not only frequently found in strains isolated from humans but also in pathogens of animal origin [53]. This is further evidence of the zoonotic character of PBIO3502.

4. Materials and Methods

4.1. Strain Origin and General Methods

As part of a small screening study, packaged fresh meat samples from various grocery stores were tested for different MDR bacteria. Briefly, the meat samples were opened under sterile conditions, two pieces of approximately 5 mm^3 were cut out with a sterile scalpel (Braun, Melsungen, Germany) and transferred to 5 mL of tryptic soy broth (Carl Roth, Karlsruhe, Germany). The bacteria were then enriched under shaking conditions (130 rpm) at 37 °C overnight. Then, 100 µL of the bacterial suspension was plated onto different chromogenic selection plates and incubated overnight at 37 °C. The strain 27-ESBL-EC (internal designation PBIO3502) was cultivated on a CHROM-ESBL selection plate (Mast

Diagnostica GmbH, Reinfeld, Germany) and the species *E. coli* was confirmed using MALDI-TOF MS (Bruker, Bremen, Germany). It was derived from a fresh bratwurst purchased from a German grocery store. All strains were stored at −80 °C in lysogeny broth (LB; Carl Roth, Karlsruhe, Germany) supplemented with 20% (*v*/*v*) glycerol (anhydrous; Merck, Darmstadt, Germany). Prior to use, one single colony of fresh overnight cultures on LB agar plates was inoculated in 5 mL of LB and grown under shaking conditions (130 rpm) at 37 °C overnight.

4.2. Whole-Genome Sequencing

Total DNA was extracted using the MasterPure DNA Purification Kit for Blood, v. 2 (Lucigen, Middleton, WI, USA), according to the manufacturer's instructions. The isolated DNA was quantified fluorometrically using the Qubit 4 fluorometer and the corresponding dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA was shipped to the Microbial Genome Sequencing Center (MiGS), now SeqCenter (Pittsburgh, PA, USA), and after library preparation using the Illumina DNA Prep Kit and IDT 10 bp UDI indices (Illumina, San Diego, CA, USA) sequenced on an Illumina NextSeq 2000, producing 2 × 151 bp reads. Demultiplexing, quality control, and adapter trimming were performed using bcl-convert v. 3.9.3 [54].

4.3. Sequence Assembly and Genomic Analyses

Short-read data were processed using BBDuk from BBTools v. 38.95 (https://sourceforge.net/projects/bbmap/, accessed on 3 March 2022) to trim the adapters, filter possible PhiX contaminants, and do further quality- and polymer-trimming. Quality control (QC) of provided (raw) and processed (trimmed) reads was performed using FastQC v. 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 3 March 2022). Trimmed reads were assembled using shovill v. 1.1.0 (https://github.com/tseemann/shovill, accessed on 3 March 2022) with SPAdes v. 3.15.3 [55]. An additional polishing step (besides the one implemented in the shovill assembly pipeline) was performed by first mapping the trimmed reads to the assembly using BWA v. 0.7.17 [56]. The alignment files were then converted to binary format, sorted, and duplicate reads were marked with SAMtools v. 1.14 [57]. Finally, the draft contigs were corrected using Pilon v. 1.24 [58]. Genome completeness and contamination were assessed using CheckM v. 1.1.3 [59]. Prokka v. 1.14.6 [60] was used to automatically annotate the draft assembly. Genomic analyses including in silico multilocus sequence typing, serotype prediction, and antibiotic resistance and virulence feature detection were performed using mlst v. 2.19.0 (https://github.com/tseemann/mlst, accessed on 3 March 2022; with the PubMLST [61] database and Enterobase [62]), ABRicate v. 1.0.0 (https://github.com/tseemann/abricate, accessed on 3 March 2022; with ResFinder [63], PlasmidFinder [10], VFDB [13], BacMet2 [64], and EcOH [65] databases), and AMRFinderPlus v. 3.10.30 with database v. 2022-05-26.1 [11]. Assignment to the phylogroup was performed using ClermonTyping v. 20.03 [66].

4.4. Phylogeny

For the creation of a core single-nucleotide polymorphism (SNP)-based phylogeny of closely related isolates, Enterobase [62] was searched for *E. coli* ST58 strains (n = 2199), for which paired Illumina read data were available (n = 2186). Data were downloaded from the public European Nucleotide Archive SRA FTP server (ftp://ftp.sra.ebi.ac.uk/vol1/, accessed on 20 June 2022). Mash v. 2.3 [67] was used to generate sketches (k-mer size, 21; sketch size, 1,000,000; minimum number of k-mers for reads, 3) of the reference sequence (draft genome of PBIO3502) and the public raw read data. Afterwards, the distance between the reference sketch and the query raw read sketches was estimated and the 200 read sets with the shortest distance (most shared k-mers) selected. For these accessions, read trimming, genome assembly, and analyses were performed as described above. The trimmed reads were mapped against the draft genome of PBIO3502 using snippy v. 4.6.0 (https://github.com/tseemann/snippy, accessed on 22 June 2022) to

create a whole-genome alignment with 201 sequences. The alignment was processed using Gubbins v. 3.2.1 [68] to filter out regions with SNPs that are likely the result of recombination. In this step, two isolates were removed due to alignment gaps or missing information at more than 25% of the alignment sites. The alignment of the remaining sequences (n = 199) was processed using snp-sites v. 2.5.1 [69] to retain only alignment positions containing A, C, G, or T exclusively. A maximum likelihood tree was inferred with RAxML-NG v. 1.1.0 [70] using GTR+G by first parsing the alignment and excluding 40 sequences that were completely identical to another sequence. The final alignment (containing 159 sequences and 4984 sites) was then processed by searching 500 parsimony and 500 random starting trees and performing 1000 bootstrap repeats. The best-scoring maximum likelihood tree was midpoint-rooted in iTOL v. 6.5.7 [71] and visualized with bootstrap support values and metadata. The exported graphic was post-processed using Affinity Designer v. 1.10.5.1342 (https://affinity.serif.com/en-us/designer/, accessed on 22 June 2022).

4.5. Minimum Inhibitory Concentration

Phenotypic antimicrobial susceptibility testing was performed in collaboration with the IMD Laboratory Greifswald (Germany) using the automated VITEK 2 system (bioMérieux, Marcy l'Etoile, France). Testing was performed using the AST-N389 card, according to the manufacturer's instructions. MIC values of gentamicin (Carl Roth, Karlsruhe, Germany), streptomycin (Sigma-Aldrich, St. Louis, MO, USA), tetracycline (Carl Roth, Karlsruhe, Germany), and tellurite (Alfa Aesar, Haverhill, MA, USA) were determined by broth microdilution according to ISO Standard 20776-1 [72]. Briefly, several single colonies were resuspended in a 0.9% (w/v) NaCl solution until the corresponding suspensions had an OD_{600} equal to 0.5 McFarland standard turbidity. The bacterial suspensions were then diluted 1:230 in cation-adjusted Mueller–Hinton broth 2 (MH-2; Sigma-Aldrich, St. Louis, MO, USA), corresponding to approximately 10^5 CFU mL^{-1}. Serial 2-fold dilutions were performed in a 96-well plate (Sarstedt, Nümbrecht, Germany) with concentrations ranging from 64 to 0.5 μg mL^{-1} for the antibiotics gentamicin, streptomycin, and tetracycline, and from 512 to 0.5 μg mL^{-1} for the heavy metal tellurite, respectively. Finally, the bacterial suspensions were added and incubated at 37 °C for 18 ± 2 h. The MIC represents the lowest concentration of antimicrobial agent that inhibits visible bacterial growth.

4.6. Biofilm Formation

Biofilm formation on polystyrene surfaces was determined by crystal violet (CV) staining, as previously described [73,74] with some adjustments. First, overnight cultures were diluted 100-fold in 5 mL of fresh M9 minimal salt medium (MP Biomedicals, Irvine, CA, USA) containing 0.4% (w/v) glucose (Carl Roth, Karlsruhe, Germany) and incubated at 37 °C and 130 rpm until OD_{600} reached 0.5 McFarland standard turbidity. The bacterial suspensions were then diluted 1:10 and 200 μL were then transferred in triplicate to a 96-well flat-bottomed polystyrene plate (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). In addition, three control wells were filled with 200 μL of sterile medium. The plates were hermetically sealed and incubated at 28 °C for 24 h without shaking. Subsequently, OD_{600} was measured using CLARIOstar Plus (BMG LABTECH GmbH, Ortenberg, Germany), planktonic cells were removed by washing three times with deionized water, and the microtiter plates were air-dried for 10 min. After fixation with 250 μL of 99% (v/v) methanol (Merck, Darmstadt, Germany) for 15 min and air-drying, the cells were stained with 250 μL of a 0.1% (w/v) aqueous CV solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The staining solution was then discarded, and unbound dye was removed by washing three times with deionized water. After air-drying for 10 min, the bound CV was dissolved with a mixture of 80 parts ethanol (99.8% (v/v); Carl Roth, Karlsruhe, Germany) and 20 parts acetone (Merck, Darmstadt, Germany) at room temperature (rt) with horizontal shaking at 200 rpm for 30 min. After complete dissolution, 125 μL were transferred to a new microtiter plate and optical density was measured at a wavelength of

570 nm using the plate reader. The strength of biofilm formation was expressed as specific biofilm formation (SBF). The SBF was calculated according to the following formula [75]: SBF = (B−NC)/G, where B is the OD_{570} of the stained bacteria, NC is the OD_{570} of the stained control wells to eliminate the fraction of CV adhering to the polystyrene surface due to abiotic factors, and G is the OD_{600} representing the density of cells grown in the media.

The expression of biofilm-associated extracellular matrix components such as cellulose and curli fibers was tested using macrocolony assays as previously described [73]. Five microliters of overnight culture was dropped onto span agar plates (Hellmuth Carroux, Hamburg, Germany). The plates were hermetically sealed and incubated at 28 °C for 5 days. For detection of curli fimbriae, 0.005% Congo red and 0.0025% Coomassie Brilliant Blue G-250 (both chemicals were purchased from Carl Roth, Karlsruhe, Germany) were added to the span agar and evaluated visually. For cellulose staining, the span agar contained 0.004% Calcofluor (Sigma-Aldrich, St. Louis, MO, USA), and the extent of cellulose formation was measured by fluorescence intensity using the plate reader (excitation, 400–415 nm, emission, 480–520 nm).

4.7. Siderophore Production Assay

Quantification of the extent of siderophores secreted by PBIO3502 was performed according to a previously published protocol [23]. Briefly, the adjusted bacterial suspensions were diluted and grown in 5 mL of chelated M9 minimal medium supplemented with casamino acids (c-M9-CA) at 37 °C and 130 rpm for 24 h. The c-M9-CA consisted of the following compounds: M9 minimal salt medium (MP Biomedicals, Irvine, CA, USA), 2 mM $MgSO_4$ (Carl Roth, Karlsruhe, Germany), 200 µM 2,2′-dipyridyl (Carl Roth, Karlsruhe, Germany), and 0.3% (*w*/*v*) casamino acids (BD, Franklin Lakes, NJ, USA). After incubation, 1 mL of the bacterial cultures were centrifuged (4900× *g* for 20 min at rt) and 100 µL of the siderophore-containing supernatant were transferred in triplicate to 96-well microtiter plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) with 100 µL of chrome azurol S shuttle solution (composition according to [76]) already in the wells. Wells containing only fresh medium and 15 mM EDTA (Carl Roth, Karlsruhe, Germany) served as a blank and positive control, respectively. The non-siderophore producer W3110 served as a negative control. All mixtures were incubated in the dark at rt for 30 min. Finally, absorbance was measured at a wavelength of 630 nm using the plate reader. Secretion of siderophores was calculated as previously described [77] and expressed as a percentage unit of siderophore production.

4.8. Serum Resistance

Determination of survival in 50% human serum was performed as previously described [23]. Briefly, overnight cultures were diluted 1:100 in 5 mL of fresh LB and incubated with shaking at 37 °C until OD_{600} reached 0.5 McFarland standard turbidity. Then, 1000 µL of the bacterial suspension was pelleted (7500× *g* for 5 min at rt) and resuspended in 1 mL of phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA). One hundred microliters of the sample were seeded in a 96-well microtiter plate containing 100 µL of human serum (United States origin; Sigma-Aldrich, St. Louis, MO, USA) per well (resulting in a final concentration of 50% human serum and approximately 10^8 CFU mL^{-1}). Following this, 20 µL of each sample was withdrawn and serial dilutions were plated on LB agar plates and incubated overnight at 37 °C to determine the size of the inoculum. The inoculated microtiter plates were incubated for 4 h at 37 °C without agitation. Thereafter, the number of surviving CFU mL^{-1} was determined by plating out serial dilutions and incubating at 37 °C overnight. The positive control in each experiment was the serum-resistant PBIO1289 (initially designated as IMT10740 [78,79]). The serum-sensitive W3110 served as the negative control. Serum resistance was expressed as $\log_2$ fold change in CFU mL^{-1} after treatment with respect to inoculum size.

4.9. Infection of Galleria mellonella Larvae

Infections of larvae of the greater wax moth *G. mellonella* were performed as previously described [23]. Overnight cultures were diluted 1:100 in 30 mL of fresh LB and incubated with shaking at 37 °C to an OD_{600} of 1.0. Then, 1000 µL of the bacterial suspension were pelleted (16,000× *g* for 5 min at rt) and washed twice with PBS. The bacterial suspensions were diluted to 10^6 CFU mL^{-1} and 10^7 CFU mL^{-1}, respectively. Larvae (proinsects, Minden, Germany) were randomly divided into groups of 10 individuals each and 10 µL of the adjusted bacterial suspensions were injected into the left proleg. In addition, 10 µL of PBS was injected into a group of larvae to ensure that death was not due to trauma from the injection. Each group was placed in 90 mm glass Petri dishes, kept at 37 °C in the dark, and death was recorded every 24 h. Individuals were considered dead when they no longer responded to physical stimuli and showed pigmentation. The results of three independent tests were pooled for each strain to generate Kaplan–Meier plots of mortality rates [80].

4.10. Data Visualization and Analysis

Data visualization was performed using GraphPad Prism v. 9.3.1 for macOS (GraphPad Software, San Diego, CA, USA). All experiments were performed with three or more independent biological replicates. Unless otherwise specified, data were expressed as mean and standard error.

5. Conclusions

The finding of a highly virulent and MDR, foodborne *E. coli* ST58, which was phylogenetically related to clinical strains of the same ST, suggests its zoonotic and pathogenic potential and the relevance of the One Health approach. Additionally, our study highlights the potential role of food (chains) in the spread and putative transmission of MDR pathogens.

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3.4 Publication 4

The study "**Extensively drug-resistant *Klebsiella pneumoniae* counteracts fitness and virulence costs that accompanied ceftazidime-avibactam resistance acquisition**" investigated how the XDR ST307 *K. pneumoniae* strain PBIO2003 acquired resistance to the drug combination ceftazidime-avibactam (CAZ-AVI) using EE. In a first EE, we selected two resistant variants by gradually increasing the concentrations of CAZ-AVI. Then, genomics, proteomics, homology modeling, and functional studies were combined to investigate the underlying resistance mechanisms. In addition, we investigated the ability of variants to counteract the associated fitness costs, again based on EE and downstream analyses. The latter included a comprehensive set of *in vitro* and *in vivo* assays combined with genomic and transcriptomic analyses.

The two initial resistant *K. pneumoniae* ST307 variants showed two different mutations in the *ompK36* gene that resulted in altered functions of the porin-encoding ortholog of *E. coli*. Decreased cellular uptake of CAZ and AVI was observed, suggesting that decreased membrane permeability due to mutation of *ompK36* leads to CAZ-AVI resistance. Next, we investigated whether mutations affecting porins in *K. pneumoniae* reduced bacterial fitness and clinically relevant properties contributing to bacterial pathogenesis. Compared to wild-type strain PBIO2003, the mutants showed significantly reduced growth, siderophore secretion, capsule formation, and resilience to bile salts and human serum. *In vivo* mortality rates in *G. mellonella* larvae were also significantly lower. Following a second EE, we investigated possible compensatory mechanisms for the initial fitness and virulence reduction. The adapted variants showed increased growth and competitive ability on a similar level as the wild-type strain, as well as improved virulence and resilience, while retaining CAZ-AVI resistance. Genomics and transcriptomics revealed mutations in the bacterial master regulator gene *rpoE* and changes in gene expression as compensatory drivers [119].

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RESEARCH ARTICLE

Extensively Drug-Resistant *Klebsiella pneumoniae* Counteracts Fitness and Virulence Costs That Accompanied Ceftazidime-Avibactam Resistance Acquisition

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ABSTRACT The ability of extensively drug-resistant (XDR) *Klebsiella pneumoniae* to rapidly acquire resistance to novel antibiotics is a global concern. Moreover, *Klebsiella* clonal lineages that successfully combine resistance and hypervirulence have increasingly occurred during the last years. However, the underlying mechanisms of counteracting fitness costs that accompany antibiotic resistance acquisition remain largely unexplored. Here, we investigated whether and how an XDR sequence type (ST)307 *K. pneumoniae* strain developed resistance against the novel drug combination ceftazidime-avibactam (CAZ-AVI) using experimental evolution. In addition, we performed *in vitro* and *in vivo* assays, molecular modeling, and bioinformatics to identify resistance-conferring processes and explore the resulting decrease in fitness and virulence. The subsequent amelioration of the initial costs was also addressed. We demonstrate that distinct mutations of the major nonselective porin OmpK36 caused CAZ-AVI resistance that persists even upon following a second experimental evolution without antibiotic selection pressure and that the *Klebsiella* strain compensates the resulting fitness and virulence costs. Furthermore, the genomic and transcriptomic analyses suggest the envelope stress response regulator *rpoE* and associated RpoE-regulated genes as drivers of this compensation. This study verifies the crucial role of OmpK36 in CAZ-AVI resistance and shows the rapid adaptation of a bacterial pathogen to compensate fitness- and virulence-associated resistance costs, which possibly contributes to the emergence of successful clonal lineages.

IMPORTANCE Extensively drug-resistant *Klebsiella pneumoniae* causing major outbreaks and severe infections has become a significant challenge for health care systems worldwide. Rapid resistance development against last-resort therapeutics like ceftazidime-avibactam is a significant driver for the accelerated emergence of such pathogens. Therefore, it is crucial to understand what exactly mediates rapid resistance acquisition and how bacterial pathogens counteract accompanying fitness and virulence costs. By combining bioinformatics with *in vitro* and *in vivo* phenotypic approaches, this study revealed the critical role of mutations in a particular porin channel in ceftazidime-avibactam resistance development and a major metabolic

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regulator for ameliorating fitness and virulence costs. These results highlight underlying mechanisms and contribute to the understanding of factors important for the emergence of successful bacterial pathogens.

KEYWORDS XDR, ST307, OmpK36, experimental evolution, fitness and virulence compensation, RpoE

Challenged by the increasing occurrence of extensively drug-resistant (XDR) (1–3) and even pan-drug-resistant (PDR) (4–6) *Klebsiella pneumoniae* strains, which are nonsusceptible to almost all classes of antibiotics (7), reliable treatment of health care- and community-associated infections caused by these pathogens poses a serious concern to the physician. The World Health Organization (WHO) ranked carbapenem-resistant *K. pneumoniae* as one of the most critical priority pathogens. This highlights the need for the development of novel antibiotics and treatment options (8). One recent example for the latter is the fixed-dose drug combination ceftazidime-avibactam (CAZ-AVI; ratio of 4:1). The United States Food and Drug Administration (FDA) approved and introduced CAZ-AVI on the market in 2015, followed by the European Medicines Agency (EMA) 1 year later (9).

While ceftazidime (CAZ) is an established third-generation cephalosporin, avibactam (AVI) is the first approved non-β-lactam β-lactamase inhibitor (10). AVI possesses potent activity against β-lactamases belonging to Ambler classes A (extended-spectrum β-lactamases [ESBL]; e.g., CTX-M-15 and *K. pneumoniae* carbapenemases [KPC]), C (AmpC β-lactamases), and D (oxacillinases; e.g., OXA-48). However, like other serine-acylating β-lactamase inhibitors, AVI cannot inhibit metallo-β-lactamases (MBL; e.g., NDM-1, Ambler class B) (11). Therefore, treating acute and life-threatening infections caused by XDR *K. pneumoniae* strains positive for both MBL and non-MBL (such as CTX-M-15 and OXA-48) by applying CAZ-AVI in combination with the only clinically available monobactam aztreonam (ATM), which is not inactivated by MBL (12), might represent the last possible therapy option. This combination was used, for example, to treat an outbreak caused by sequence type (ST)307 *K. pneumoniae* in different health care institutions in Western Pomerania (Germany) (1, 13) and an outbreak by ST147 *K. pneumoniae* in a hospital in Barcelona (Spain) (14). Studies have previously reported CAZ-AVI and ATM-AVI resistances in *Enterobacterales*. The respective strains showed decreased membrane permeability through changes of outer membrane proteins (OMPs) (15), induction of efflux (16), modification of the targeted penicillin-binding protein 3 (PBP3) (17), or point mutations in active sites of β-lactamases (e.g., Lys237Gln of CTX-M-15 [18], Pro68Ala in combination with Tyr211Ser of OXA-48 [19], and Ala172Thr of KPC-3 [20]). The broad genetic diversity and range of known and unknown mechanisms of antimicrobial resistance acquisition underline the continued need for the further and in-depth characterization of resistance determinants.

In addition to "classical" carbapenem-resistant *K. pneumoniae* (cKp) described above, there is the hypervirulent *K. pneumoniae* (hvKp) pathotype (21–23). The latter is defined by its general susceptibility to antibiotics, the community association of infections caused, and high-level virulence, which is characterized phenotypically by hypermucoviscosity and extensive siderophore secretion (24–27). Clinically, infections caused by hvKp are often invasive and include, for instance, pyogenic liver abscess, pneumonia, endophthalmitis, meningitis, necrotizing fasciitis, and bacteremia (22). Alarmingly, the convergence of both cKp and hvKp has already been frequently described (1, 2, 28), which blurs the boundaries between the pathotypes. This phenomenon is seemingly driven not only by the exchange of resistance (29–31) and virulence plasmids (28, 32) but also by the chromosomal integration of DNA sequences with uncommonly high length (23, 33–35) as well as rearranged "mosaic" plasmids that carry both resistance- and virulence-associated genes (1, 36–38). We have previously reported on such mosaic plasmids, which do not necessarily reduce bacterial fitness, in the above-mentioned ST307 *K. pneumoniae* clonal lineage (1). Thus, this apparent ability of bacterial pathogens to improve their fitness

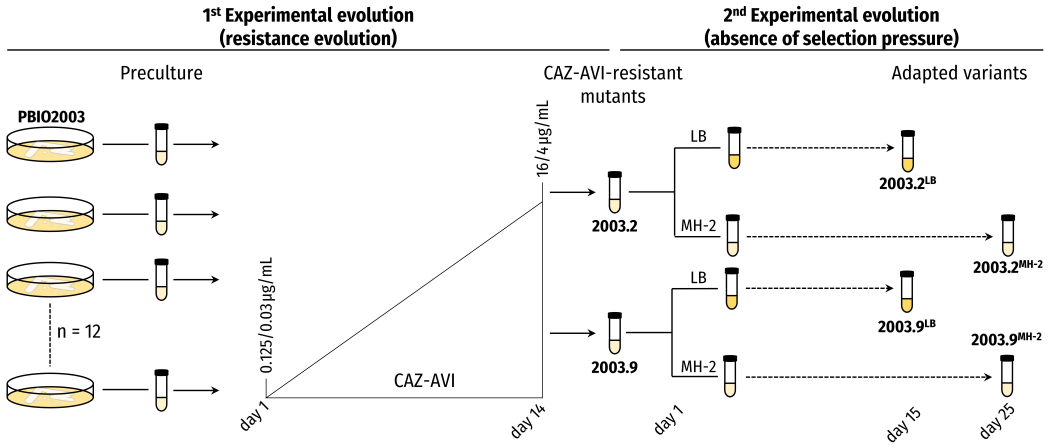

FIG 1 Schematic presentation of the experimental design. To investigate the resistance acquisition against CAZ-AVI, we inoculated 12 randomly chosen single colonies of PBIO2003 individually in 1 mL of MH-2 and incubated them overnight (preculture). Then, the stationary-phase cultures were transferred daily in the presence of increasing CAZ-AVI concentrations until some replicates tolerated concentrations of 16/4 μg/mL CAZ-AVI. We then used a second EE approach to investigate compensatory events overcoming (putative) fitness burdens. One population, each of 2003.2 and 2003.9, was propagated in LB or MH-2, and an everyday stationary-phase culture was transferred into fresh medium.

through plasmid adaptation and compensatory mutations might explain the success of some clonal lineages (39). However, due to the redundancy and epistasis of genes and complexities of underlying pathways (40, 41), little is known about how and which mutations and regulatory mechanisms counteract fitness reduction (and associated virulence) caused by antimicrobial resistance acquisition (39).

Here, we investigated (i) the acquisition of resistance against CAZ-AVI as well as cooccurring resistances of a clinical *K. pneumoniae* strain and (ii) underlying mechanisms following an experimental evolution (EE) approach. Then, we explored (iii) whether and how this resistance acquisition might be compensated, again based on EE and downstream analyses. Overall, by combining a comprehensive set of *in vitro* and *in vivo* experiments with genomic and transcriptomic analyses, we reveal deep insights into the resistance evolution against CAZ-AVI and accompanied compensatory mechanisms to ameliorate fitness and virulence costs in XDR *K. pneumoniae*.

RESULTS

Increased tolerance against CAZ-AVI and ATM due to reduced membrane permeability. The emergence of XDR *K. pneumoniae* has been frequently described (1–3). However, we do not yet fully understand the underlying mechanisms and dynamics of resistance acquisition, especially against newer antibiotics and drug combinations. One objective of this study was thus to investigate resistance development against CAZ-AVI. We used a well-characterized XDR, yet CAZ-AVI-sensitive, ST307 *K. pneumoniae* strain (PBIO2003) (1) obtained from the outbreak mentioned earlier that took place in four different health care institutions in Western Pomerania (Germany) in 2019 and the beginning of 2020. Compared to the other ST307 isolates from the outbreak, PBIO2003 had lost the $bla_{\text{NDM-1}}$ gene and, therefore, showed susceptibility to CAZ-AVI and CAZ-AVI combined with ATM.

Originally, we started with 12 biological replicates and a concentration of 0.125/0.03 μg/mL CAZ-AVI (one-fourth MIC; Fig. 1). With daily increasing concentrations of up to 16/4 μg/mL CAZ-AVI (which is considered resistant according to the European Committee on Antimicrobial Susceptibility Testing [EUCAST] guidelines [42]), we obtained overall two resistant replicates (16.67%, 2/12) within 14 days.

When comparing the CAZ-AVI-resistant mutants (subsequently termed 2003.2 and 2003.9) with the genome of the ancestral wild-type strain (PBIO2003), our analysis revealed different mutations in the *ompK36* gene (Fig. 2). This gene encodes an OMP

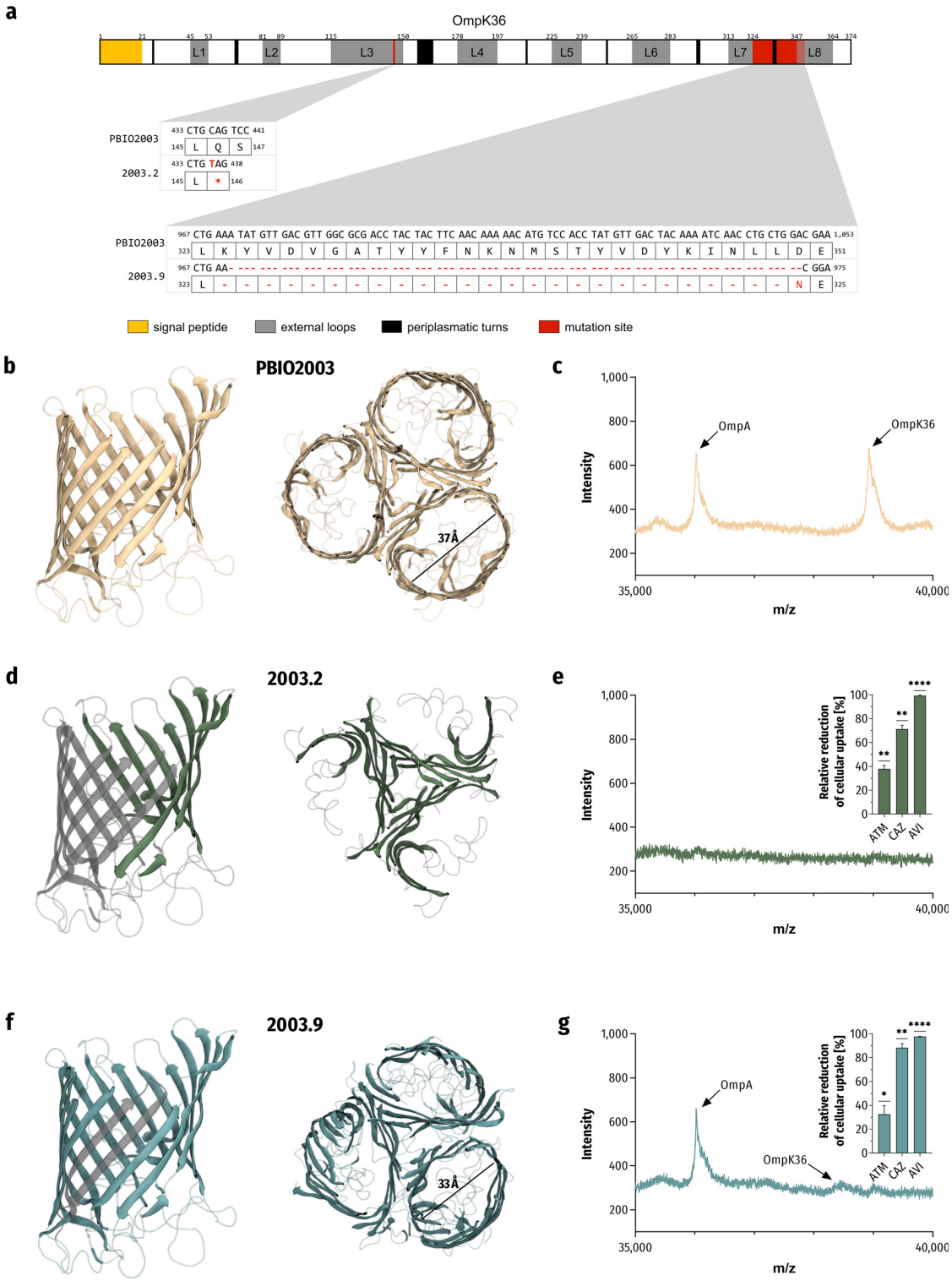

FIG 2 Different mutations of *ompK36* change the outer membrane proteins' architecture and reduce CAZ, AVI, and ATM uptake. (a) Schematic presentation of genetic changes in the *ompK36* gene of the CAZ-AVI-resistant mutants 2003.2 and 2003.9. The different mutations are marked

(Continued on next page)

(designated porin) orthologue of *Escherichia coli* OmpC (43). Porins are β-barrel proteins composed of antiparallel β-sheets, thus forming an essential aqueous transmembrane channel system (44). For 2003.2, we found a nonsense single-nucleotide polymorphism (SNP) of *ompK36* (436C>T [Gln146*]; Fig. 2a), resulting in a premature stop codon and ultimately truncated translation, which usually leads to a nonfunctional protein. To further explore the molecular effects of this mutation, we predicted the protein structure of OmpK36 by homology modeling (Fig. 2b, d, and f). As shown in Fig. 2d, the protein structure model of the premature stop codon mutation supported our prediction regarding loss of function due to the absence of large parts of the porin channel transmembrane region. Additionally, we identified a read-through mutation in *dsbA* (624A>T [*208Tyr]) with a putative in-frame C terminus extension of 16 amino acids in 2003.2 that may lead to changes in protein expression levels and stability (45). This gene encodes a thiol-disulfide interchange protein, typically required for disulfide bond formation in periplasmatic proteins such as OmpA (46, 47). The second mutant (2003.9) showed a fragment deletion 78 bp in length (corresponding to 26 codons), resulting in an intact reading frame with a predicted deletion of transmembrane β-sheets between the external loops L7 and L8 and a reduced pore diameter (Fig. 2b and f). Note, however, that the structure generated for 2003.9 does not represent a complete atomistic model but is instead used to visualize a significant decrease of the β-barrel diameter, explaining the possible resistance mechanism.

We then used a matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) approach to identify the expressed outer membrane proteins and evaluate our predicted phenotypes (Fig. 2c, e, and g). Mutant 2003.2 (premature stop codon mutation; Fig. 2e) revealed a lack of the OmpK36 signal (m/z of 38,933). Interestingly, we also obtained no signal associated with the OmpA channel (m/z of 36,026). The β-strand-deleted variant (2003.9; Fig. 2g) showed a shift of the OmpK36 signal based on mass loss (m/z of 38,465). Notably, both the wild-type strain PBIO2003 (Fig. 2c) and mutants (2003.2 and 2003.9) showed only a low coverage of the *ompK35* annotation. This was consistent with our findings in the MALDI-TOF MS examination of the outer membrane proteins ($m/z_{expected}$ of 37,926).

It is well known that cross-resistance (48) and collateral sensitivity (49) may accompany resistance acquisition. To further investigate the effect of the aforementioned mutations on the antibiotic resistance profile, we determined MICs of a large panel of relevant antibiotic drugs and combinations (Table S1 in the supplemental material). Phenotypic antimicrobial susceptibility testing (AST) revealed for 2003.2 and 2003.9 a strong increase not only of CAZ-AVI MIC values (>64/4 μg/mL CAZ-AVI) but also of CAZ-AVI in combination with ATM (>64/4/64 μg/mL CAZ-AVI/ATM). Interestingly, we found no evidence of collateral sensitivity as described previously in another study (50). We then analyzed potential differences in the compound uptake of each of the triple combination of the β-lactam antibiotics ATM and CAZ and the non-β-lactam β-lactamase inhibitor AVI. Therefore, we used a high-resolution continuum-source molecular absorption spectrometry (HR CS MAS) approach to determine changes in endogenous sulfur content following treatment with the sulfurous compounds (Fig. 2e and g). Both mutants showed similar trends of relative uptake reductions compared to PBIO2003, whereby the uptake of CAZ and AVI seemed highly affected by the reduced outer membrane permeability. However, while the uptake of CAZ was significantly reduced by up to 71.3% (PBIO2003 versus 2003.2: $P = 0.0019$) and 88.4% (PBIO2003 versus 2003.9: $P = 0.0014$), respectively, ATM uptake was reduced by only about one-third (PBIO2003 versus 2003.2: 37.9%, $P = 0.0012$;

FIG 2 Legend (Continued)
at their respective positions (red). (b, d, and f) Cartoon representation of modeled protein structures of the trimeric OmpK36 of PBIO2003 (wild-type; b), 2003.2 (premature stop codon; d), and 2003.9 (deletion of β-sheets between the external loops L7 and L8; f). Predicted changes in the architecture of the porin channel in lateral view (left) are colored in transparent gray. (c, e, and g) Mass spectra of outer membrane proteins represent differences in the configuration of expressed proteins of 2003.2 (e) and 2003.9 (g) compared to PBIO2003 (c). The insets show that changes in membrane permeability reduce CAZ, AVI, and ATM uptake into 2003.2 and 2003.9. The results are given as mean values of percent relative reduction related to PBIO2003 and standard error ($n = 3$). The results were analyzed using a one-sample t test, and the following indicate the significance level (P value): *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

PBIO2003 versus 2003.9: 32.6%, $P = 0.0450$). The uptake of AVI was almost no longer measurable (PBIO2003 versus 2003.2: 99.3%, $P < 0.0001$; PBIO2003 versus 2003.9: 97.6%, $P < 0.0001$), thus supporting the role of OmpK36 in resistance acquisition against CAZ-AVI and ATM.

In summary, our results suggest that the decreased membrane permeability through mutation of the *ompK36* gene conferred resistance against CAZ-AVI and CAZ-AVI/ATM. Moreover, similar changes in MICs and comparable reduction in compound uptake indicate that the expression of OmpK36 with reduced pore diameter (2003.9) is as effective as the complete deletion of the porin (2003.2). In addition, we show that the uptake of the bridged 1,6-diazabicyclo[3.2.1]octan-7-one derivative AVI is highly dependent on OmpK36, which may suggest cross-resistance to other members of this class of diazabicyclooctanes β-lactamase inhibitors (e.g., nacubactam or zidebactam).

Membrane impermeability negatively affects growth, virulence, resilience, and mortality. Although porin changes conferring reduced membrane permeability have been frequently described, those mutants rarely cause outbreaks (18). This might be explained by the fact that modification of nonspecific porins leads to a fitness reduction and/or, subsequently, a decrease of virulence (51–55). This is why we challenged the porin mutants 2003.2 and 2003.9 in phenotypic experiments, including growth, virulence, resilience, and *in vivo* mortality, to investigate whether these mutations influence clinically relevant features important for bacterial pathogenesis (Fig. 3).

We observed significantly decreased growth behaviors in LB for both mutants compared to wild-type PBIO2003 (area under the growth curves [AUC] of 60.61; Fig. 3a and b), with 2003.2 (AUC of 39.72, $P < 0.0001$) showing a higher decrease than 2003.9 (AUC of 50.08, $P < 0.0001$).

We next determined siderophore secretion and mucoid phenotypes to investigate whether the growth decrease was associated with a lower virulence level (Fig. 3c and d). The biosynthesis and secretion of siderophores, which are small iron-chelating compounds that seize iron from the host (56), play a crucial role during bacterial infection and are hallmarks of hypervirulent *K. pneumoniae* (57). Our analysis revealed a significant decrease of siderophore secretion for 2003.2 (48.3%, $P < 0.0001$) and 2003.9 (67.3%, $P = 0.0102$) compared to PBIO2003 (71.3%; Fig. 3c). Another important virulence-associated feature of pathogenic *K. pneumoniae* is capsule formation, which functions as a physiological barrier and conveys protection against the hosts' immune system (e.g., protection from phagocytosis) (58, 59). Hypermucoviscosity quantification is based on prolonged sedimentation and the following retaining of mucus in the supernatant after centrifugation of hypermucoviscous cells (60). Here, the mutant cells almost entirely sedimented and formed a tighter pellet than the wild-type cells (PBIO2003: 0.225), thus indicating decreased mucoviscosity (Fig. 3d). Again, we noticed a higher decrease in this virulence-associated feature in 2003.2 (0.061, $P < 0.0001$) than in 2003.9 (0.134, $P = 0.0001$).

Since *K. pneumoniae* is a leading cause of pyogenic liver abscesses (61–64) and bacteremia (65, 66), its resilience against bile salts and serum seems essential for pathogenesis. Therefore, and to investigate the impact of reduced membrane permeability on bacterial stress response and resilience, we challenged the mutants with complement-containing human serum and bile salts for 4 h each. We observed a significant decrease in survival of the OmpK36 mutants in response to both external stressors, which indicates reduced pathogenicity of 2003.2 and 2003.9 compared to PBIO2003 (Fig. 3e and f).

We finally performed a larvae infection model of the greater wax moth *Galleria mellonella* to study the mutants' ability to cause infection-associated mortality *in vivo* (67). We injected equivalent numbers of CFU of the wild-type and both OmpK36 mutants in the right proleg and monitored the death of larvae every 24 h. When injecting 2×10^4 CFU of PBIO2003 per larvae, we detected a mortality rate of 13.3% after 24 h and 46.7% after 72 h of incubation (Fig. 3g). When using the same inoculation size of 2003.2 and 2003.9, we observed 100% larval survival 72 h after injection. Mortality rates increased slightly when higher concentrations of the

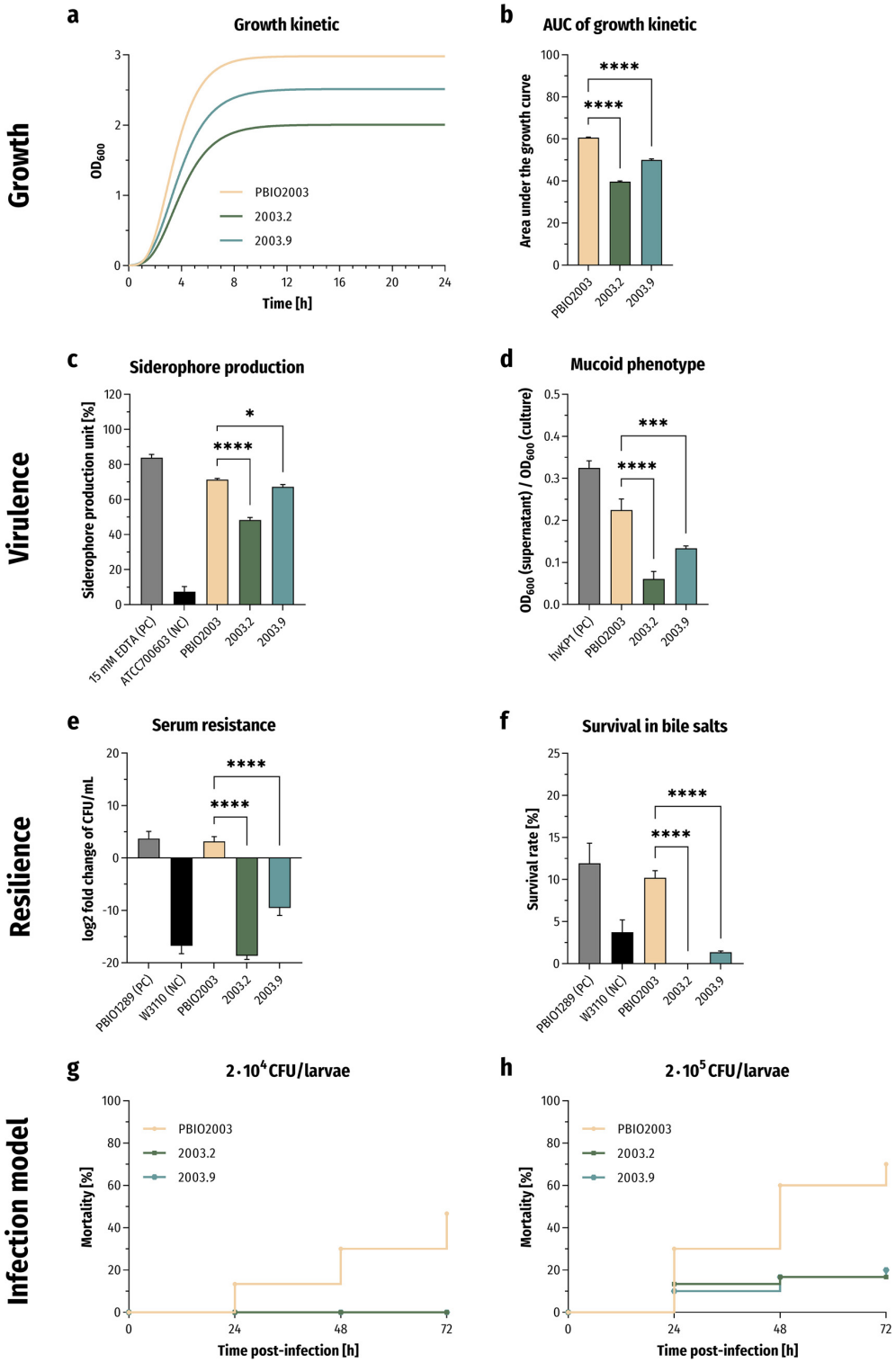

FIG 3 Phenotypic characteristics of the CAZ-AVI-resistant mutants 2003.2 and 2003.9. (a and b) Gompertz growth-fitting curves of the growth kinetics in LB (n = 3; a) and statistical comparison of area under the growth curves (AUC; b). The results are

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mutants (2×10^5 CFU) were used, but they remained significantly lower than for the wild-type (Fig. 3h).

Adaptative mechanisms increase fitness, virulence, and resilience while resistance is retained. While previous studies frequently showed that *ompK36* mutations reduce fitness (51–55), it remains mostly unclear whether and how exactly this might be compensated. Ameliorating these costs may also play a crucial role in the emergence and maintenance of resistance within the bacterial population (68), even without antibiotic selection pressures. We thus performed a second EE experiment and evolved both 2003.2 and 2003.9 mutants (Fig. 1) to explore potential compensatory events that occur independently of antibiotic selection pressures. Since different growth conditions might directly affect evolutionary adaptation (69, 70), we used two complex media to increase the chance of identifying actual beneficial mutations. The first was LB medium, which contains no fermentable sugars and primarily provides amino acids as a carbon source (71). The second was cation-adjusted Mueller-Hinton broth 2 (MH-2), which is formulated for antimicrobial susceptibility testing according to EUCAST guidelines (42).

During the second EE, we tracked the growth behaviors of each population every fifth day. Noticeably, after 15 days of serially passaging in LB and 25 days in MH-2, a fitness behavior of the evolved variants comparable to the growth of wild-type PBIO2003 was observed (Fig. 4a and b). Hence, compared to their parental strain 2003.2 (competitive index [CI] of 0.0060), the adapted variants 2003.2^{LB} (CI of 0.4480, $P = 0.0002$) and $2003.2^{MH\text{-}2}$ (CI of 0.1547, $P = 0.0344$) showed a significant increase in growth and competition. However, 2003.9^{LB} (CI of 0.2041, $P = 0.2505$) and $2003.9^{MH\text{-}2}$ (CI of 0.2117, $P = 0.2230$) exhibited only weak changes in their growth behaviors compared to the parental mutant 2003.9 (CI of 0.0647).

We hypothesized that fitness recovery is accompanied by an increase in virulence and resilience. We thus tested the adapted variants in our set of phenotypic experiments (Fig. 4c to f). Compared to the parental strain 2003.2, the adapted variants 2003.2^{LB} and $2003.2^{MH\text{-}2}$ showed a significant increase in siderophore secretion (Fig. 4c; 2003.2 48.3% and 2003.2^{LB} 84.7%, $P < 0.0001$; $2003.2^{MH\text{-}2}$ 83.9%, $P < 0.0001$) and mucoviscosity (Fig. 4d; 2003.2 0.061 and 2003.2^{LB} 0.182, $P = 0.0003$; $2003.2^{MH\text{-}2}$ 0.155, $P = 0.0013$). In addition, we observed a significant increase in resilience toward human serum and bile salts (Fig. 4e and f). In contrast, changes in the adapted variants 2003.9^{LB} and $2003.9^{MH\text{-}2}$ compared to their parent 2003.9 seemed slightly different. Again, we obtained a significant increase in siderophore production (Fig. 4c; 2003.9 67.3% and 2003.9^{LB} 84.4%, $P = 0.0003$; $2003.9^{MH\text{-}2}$ 84.2%, $P = 0.0003$) and mucoviscosity (Fig. 4d; 2003.9 0.134 and 2003.9^{LB} 0.207, $P = 0.0044$; $2003.9^{MH\text{-}2}$ 0.261, $P = 0.0003$), respectively. However, only 2003.9^{LB} showed a significant increase in serum resistance (Fig. 4e), and, interestingly, 2003.9^{LB} and $2003.9^{MH\text{-}2}$ showed a significant decrease in tolerance against bile salts (Fig. 4f) compared to 2003.9. Notably, the *G. mellonella* larvae infection model revealed increased mortality caused by each adapted variant originating from 2003.2 and 2003.9. By injecting 2×10^5 CFU of 2003.2^{LB} and $2003.2^{MH\text{-}2}$ per larvae, respectively, we detected mortality rates of 20.0% (2003.2^{LB}) and 26.7% ($2003.2^{MH\text{-}2}$) after 24 h and 56.7% (2003.2^{LB}) and 40.0% ($2003.2^{MH\text{-}2}$) after 72 h of incubation (Fig. 4g). When using the same inoculation volume of 2003.9^{LB} and $2003.9^{MH\text{-}2}$ (Fig. 4h), we observed mortality rates of 30.0% (2003.9^{LB}) and 23.3% ($2003.9^{MH\text{-}2}$), resulting in overall mortality rates of 56.7% (2003.9^{LB}) and 40.0% ($2003.9^{MH\text{-}2}$) after 72 h.

FIG 3 Legend (Continued)
given as mean values and standard deviation of AUCs. (c) The extent of secreted siderophore is presented as mean values of the siderophore production unit and standard deviation ($n = 3$). (d) Determination of mucoid phenotype using a sedimentation assay ($n = 3$). The results are given as mean ratios of OD_{600} of supernatant after centrifugation at 1,000 $\times$ *g* for 5 min and total OD_{600} and standard deviation. (e) Survival in 50% human serum ($n = 3$). The results are given as mean values and standard deviation of $\log_2$ fold change of CFU/mL after 4 h of incubation in the presence of serum. (f) Resilience against 50 mg/mL bile salts ($n = 3$). The results are shown as mean percent survival rates and standard deviation. (g and h) Kaplan-Meier plot of mortality rates in the *Galleria mellonella* larvae infection model ($n = 3$). The results are given as mean percent mortality following injection of 2×10^4 CFU/larvae (g) and 2×10^5 CFU/larvae (h). For all results, the mutants were compared to PBIO2003 using variance analyses (one-way ANOVA with Dunnett's multiple comparison *post hoc* test); *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

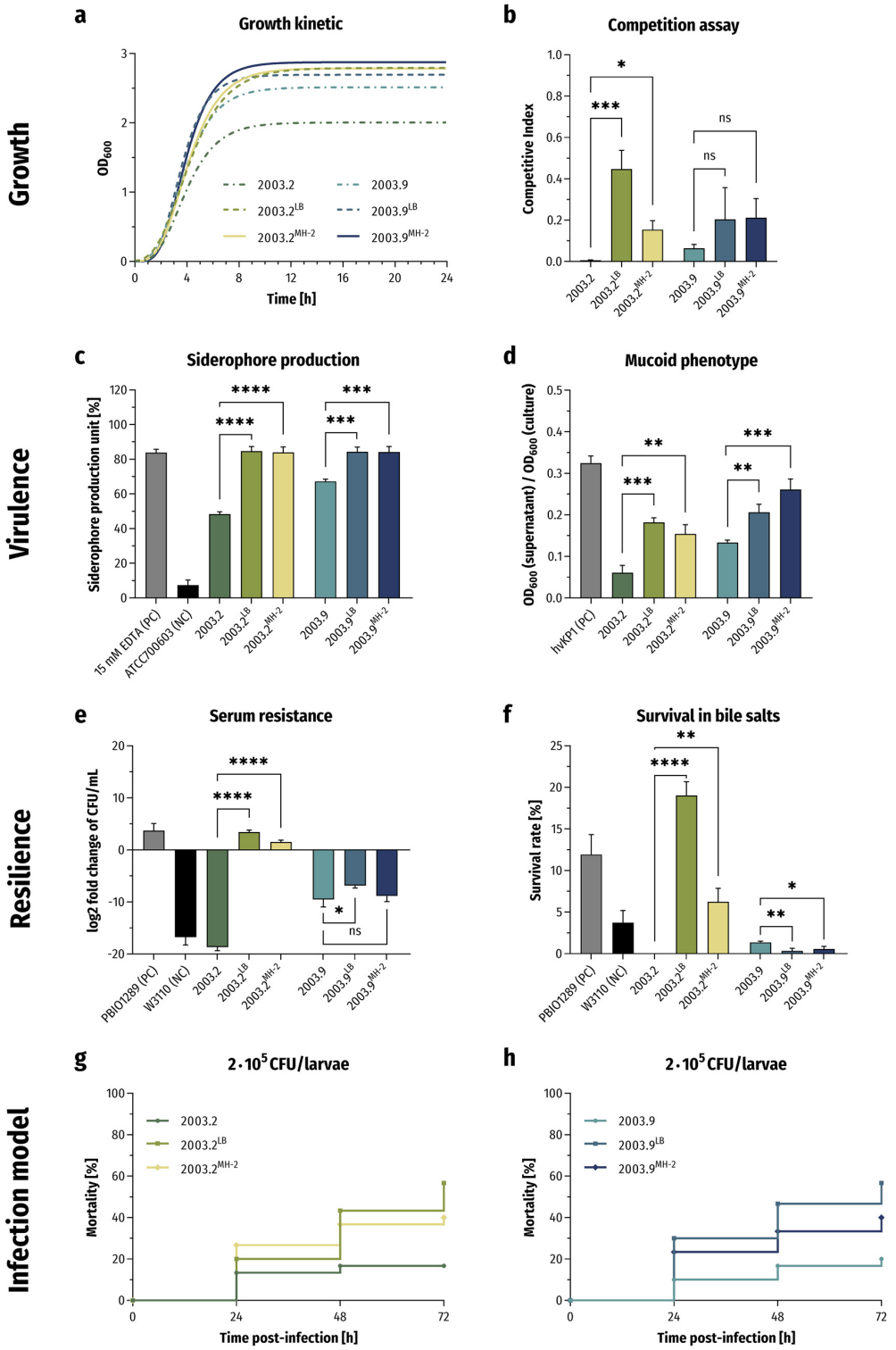

FIG 4 Phenotypic changes of the adapted variants of 2003.2 and 2003.9. (a) Gompertz growth-fitting curves of the growth kinetics in LB (n = 3). (b) Quantification of competitive interactions. The results are given as mean values and standard deviation

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In summary, with the second EE experiment, we obtained adapted variants that seemingly restored their fitness and virulence compared to the original wild-type strain. To assess the underlying mechanisms of these compensatory events, we next performed genomic analyses. When comparing the genomes of 2003.2^{LB} and 2003.2^{MH-2} with the parental strain 2003.2, our analysis revealed that the premature stop codon in *ompK36* and the read-through mutation of *dsbA* were still present. Moreover, we identified two different missense mutations in *rpoE* (2003.2^{LB}: 154G>A [Glu52Lys]; 2003.2^{MH-2}: 97G>T [Val33Phe]), encoding one of the primary regulators of the enveloped stress response system, which is activated through accumulated misfolded proteins and lipopolysaccharide (LPS) fragments in the periplasmatic space (72, 73). Upon alternative sigma factor E (σ^E) triggering, it forms a holoenzyme with the bacterial core RNA polymerase complex (RNAP) and initiates transcription of more than 100 protein-coding genes, such as proteins required for DNA recombination and repair, lipid A biosynthesis, and LPS translocation as well as OMP membrane insertion (74, 75). Interestingly, both missense mutations were located in the highly conserved domain σ^E_2, which stabilizes the transient association of σ^E with the RNAP, thus forming the active holoenzyme and initiating transcription (76). Therefore, these *rpoE* mutations might destabilize the RNAP binding, resulting in decreased transcription levels of RpoE-regulated genes. Furthermore, this might reduce the ability of σ^E to compete with the other sigma factors for RNAP (77).

In contrast to the variants of 2003.2, when comparing the genomes of 2003.9^{LB} and 2003.9^{MH-2} with 2003.9, we only identified the expected deletion of transmembrane β-sheets between the loops L7 and L8 of *ompK36* but no other mutations.

Pathogenic bacteria rapidly respond to surrounding changes during different stages of infection by transcriptomic regulation (78, 79). These complex regulatory networks not only protect the cell from external stressors but also improve fitness and the expression of virulence-associated features such as enhanced iron uptake (79, 80). While costly genetic modifications in the *ompK36* gene in the presence of CAZ-AVI represent an advantageous trade-off, reduced membrane permeability in the absence of antibiotics itself is a stressor that resistant strains must cope with. This might be reflected by significant transcriptomic changes to compensate for fitness costs and overcome high cellular stress levels. This is why we next examined the transcriptomes of the adapted variants 2003.2^{LB}, 2003.2^{MH-2}, 2003.9^{LB}, and 2003.9^{MH-2} compared with their parental strains 2003.2 and 2003.9, respectively (Fig. S1 to S3 in the supplemental material). The Clusters of Orthologous Groups (COG) analysis revealed an upregulation of genes encoding cell motility and extracellular structures in all adapted variants (Fig. S4 and S5 in the supplemental material). However, the adapted variants showed extensive downregulation of genes associated with information storage and processing, cellular processes and signaling, and metabolism. Interestingly, differential gene expression (DGE) analysis of 2003.2^{LB} and 2003.2^{MH-2} compared with 2003.2 revealed significant downregulation of genes whose transcription is induced by σ^E, thus supporting our hypothesis that both missense mutations of *rpoE* resulted in a loss of function of this alternative sigma factor (Fig. S4 in the supplemental material). These downregulated σ^E regulon genes are required for proper folding and assembly of OMPs (*bamACDE*, *fkpA*, *skp*), phospholipid and LPS biogenesis and modification (*eptB*, *lpxP*, *phoP*), cellular processes and regulation (*csrD*, *cutC*, *htrA*, *rpoE*, *rseP*), as well as

FIG 4 Legend (Continued)
of competitive indices (CIs). (c) The extent of secreted siderophore is presented as mean values of the siderophore production unit and standard deviation ($n = 3$). (d) Determination of mucoid phenotype by using a sedimentation assay ($n = 3$). The results are given as mean ratios of OD_{600} of supernatant after centrifugation at 1,000 $\times$ *g* for 5 min and total OD_{600} and standard deviation. (e) Survival in 50% human serum ($n = 3$). The results are given as mean values and standard deviation of $\log_2$ fold change of CFU/mL after 4 h of incubation in the presence of serum. (f) Resilience against 50 mg/mL bile salts ($n = 3$). The results are shown as mean percent survival rates and standard deviation. (g and h) Kaplan-Meier plot of mortality rates in the *Galleria mellonella* larvae infection model ($n = 3$). The results are given as mean percent mortality following injection of 2×10^5 CFU/larvae. For all results, the adapted variants were compared to their respective parental strain using variance analyses (one-way ANOVA with Dunnett's multiple comparison *post hoc* test); ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

unknown functions (*yfeXY*) (75, 81). Furthermore, we noticed an upregulation of genes encoding porins (*ompA*, *ompK36*), which is usually associated with the inactivation or repression of σ^E during exponential growth (74). Taken together, these results strongly support the role of RpoE suppression in regaining fitness and virulence as shown by 2003.2^{LB} and 2003.2$^{MH\text{-}2}$ results. Also note that we detected a significant increase of *ompK17* expression for both 2003.2^{LB} and 2003.2$^{MH\text{-}2}$. Various studies reported that this small OMP, which is a structural homolog of OmpX of *E. coli* (82), is involved in cell adhesion and biofilm formation (83) and contributes to resistance against complement-mediated killing and *in vivo* mortality (84), thus possibly also participating in the restored phenotypes of 2003.2^{LB} and 2003.2$^{MH\text{-}2}$.

The DGE analysis of 2003.9^{LB} and 2003.9$^{MH\text{-}2}$ compared to their parental strain 2003.9 revealed a significant upregulation of *nhaA* (Fig. S5 in the supplemental material), which encodes an essential sodium proton antiporter involved in enhanced pH tolerance and sodium and volume homeostasis, crucial to cell viability (85). Interestingly, a previous study showed that the deletion of *nhaA* leads to severe attenuation of virulence in pathogenic *E. coli* strains in mammalian and avian infection models (86). Moreover, an orthologue of NhaA expressed by *Yersinia pestis* contributes to bacterial survival in the bloodstreams of infected mice (87), thus indicating that NhaA might contribute to fitness and virulence regain of 2003.9^{LB} and 2003.9$^{MH\text{-}2}$. In addition, we detected a significant downregulation of *dksA*, an RNA polymerase-binding transcription factor, which is a part of the stringent response. Under nutrition-limited conditions, bacteria must adjust their metabolic activity and growth to maintain a balance between cell survival and proliferation. Therefore, they synthesize signaling nucleotides (ppGpp) that interact with two binding sites of RNAP, leading to transcription initiation. DksA binds to another binding site of RNAP and modulates its activity in conjunction with ppGpp (88). Additionally, in the absence of ppGpp, DksA regulates rRNA transcription initiation. A previous study demonstrated that the rRNA promoter activity of a Δ*dksA* mutant does not decrease following entry into the stationary phase, and the promoter does not respond to changes in growth rates or amino acid starvation (89). This might be another explanation for the fitness and virulence recovery of 2003.9^{LB} and 2003.9$^{MH\text{-}2}$.

Finally, we verified that the resistant phenotypes were still present in 2003.2^{LB}, 2003.2$^{MH\text{-}2}$, 2003.9^{LB}, and 2003.9$^{MH\text{-}2}$ by again examining the MIC of a large panel of relevant antibiotic drugs and combinations (Table S1). Our analysis revealed that most resistance profiles of 2003.9^{LB} and 2003.9$^{MH\text{-}2}$ strains were similar to their parental strain 2003.9. In contrast, we detected a difference regarding the last-resort antibiotic colistin in 2003.2^{LB} and 2003.2$^{MH\text{-}2}$ (from 16 μg/mL to 0.5 μg/mL). It is known that incorporation of phosphoethanolamine and 4-amino-4-deoxy-L-arabinose in lipid A lowers the negative charge of the bacterial LPS, which subsequently leads to resistance (90). Note that here, the aforementioned decrease of RpoE activity in 2003.2^{LB} and 2003.2$^{MH\text{-}2}$ leads to reduced expression of genes related to these lipid A modifications (*eptB*, *lpxP*, *phoP*), resulting in exposure of the negative charge and consequent colistin susceptibility.

In summary, here we show that a resistance-accompanying decrease in fitness and virulence was compensated following bacterial evolution in the absence of antibiotic selection pressure, while CAZ-AVI resistance was still present. Our genomic and transcriptomic analyses suggest that mutations in *rpoE* and expression differences of RpoE-regulated genes were drivers of this compensation along with, however, a loss of colistin resistance due to alterations of LPS.

DISCUSSION

Although combinations of synergistically interacting drugs (such as combinations of β-lactams and β-lactamase inhibitors) are often used to treat infections, Hegreness et al. (91) showed that these combinations did not sufficiently suppress resistance acquisition. The authors concluded that mutations confer simultaneous resistance against both compounds. In particular, sublethal antimicrobial concentrations hereby appear to induce rapid mutagenesis (92). Starting with a subinhibitory concentration and daily increasing

CAZ-AVI concentrations, we obtained two different CAZ-AVI-resistant mutants within 14 days only. Although this approach does not exactly reflect a guideline-oriented therapy in clinical practice (93), the rapid resistance development is remarkable and underlines the clinical importance of our finding.

As mentioned before, combining ATM with CAZ-AVI to treat bacterial pathogens that harbor both MBL and non-MBL is a last-resort drug possibility. Since a fixed-dose product of ATM-AVI is currently unavailable, increased tolerance of pathogens against this combination has been rarely described clinically (94). Quite recently, Nordmann et al. (95) reported the occurrence of *E. coli* strains showing phenotypic nonsensitivity against ATM-AVI, however, conferred by modifications of PBP3 with cooccurrence of different β-lactamases. Our study provides evidence of mutations in *ompK36* following EE of a clinical XDR *K. pneumoniae* strain, resulting in both resistance against CAZ-AVI and nonsensitivity against CAV-AVI with ATM. We show that these mutations resulted phenotypically in nonexpression of OmpK36 (2003.2, premature stop codon) and reduced pore diameter (2003.9, deletion of transmembrane β-sheets between the loops L7 and L8), respectively. Previous studies associated alterations of OmpK35/36 channels with CAZ-AVI resistance in *K. pneumoniae* in clinical settings (96–98), but, due to the complexities in underlying genetic mechanisms, interactions of specific β-lactamases and the diversity of *ompK35/36* variants, their potency in resistance contribution remains controversial (99). For example, Pagès et al. (100) demonstrated that ESBL-producing *K. pneumoniae*, which additionally lacked one or both OmpK porins, had increased MIC values of CAZ but were still susceptible to CAZ-AVI. Hence, the authors concluded that OmpK35 and OmpK36 did not contribute to the intracellular uptake of AVI through the outer membrane. In contrast, we unequivocally verified that the combination of missing OmpK35 expression and changes in the OmpK36 channel led to almost entirely reduced uptake of the third-generation cephalosporin CAZ and the bridged 1,6-diazabicyclo[3.2.1]octan-7-one derivative AVI. Interestingly, this seems to be independent of the expression of OmpA, as 2003.9 showed similar or sometimes higher relative uptake reductions, although we detected that OmpA was expressed. Moreover, the similarity of MIC changes and reduction in compound uptake indicates that the expression of OmpK36 with reduced pore diameter is as effective as the complete deletion of the porin. Results of another study support this finding (55). Consistent with the findings of our study, the authors demonstrated that two mutation types of OmpK36 had comparable potency in the contribution of β-lactam resistance. Furthermore, OmpK35 was shown to play only a minor role in this resistance propagation; an issue that we were not able to investigate more closely due to the intrinsic absence of OmpK35 in the wild-type strain PBIO2003. Overall, our results demonstrate the importance of OmpK36 in rapid resistance acquisition.

It is well-known and has been frequently described that resistance acquisition is, directly and indirectly, accompanied by a reduction of bacterial fitness and virulence (101). In fact, our porin-mutated strains 2003.2 and 2003.9 showed significantly decreased growth behaviors compared to wild-type PBIO2003. Moreover, we found a strong decrease in virulence-associated features and stress resilience, as also demonstrated by reduced *in vivo* mortality. Consistent with our findings, previous studies have reported a fitness reduction and lack of virulence of strains that obtain OMP alterations (51–55), thus indicating the key role of porins in maintaining fitness and consequently virulence. Overall, our results suggest that antibiotic resistance acquisition is a double-edged sword. On the one side, it is necessary for survival in environments with respective selection pressures. On the other side, resistance acquisition comes at a cost and may lead to reduced fitness, virulence, resilience, and mortality, likely resulting in decreased competition potentials and a lack of features required for the successful adaptation to new niches and pathogenicity. Interestingly, our genomic analysis showed that the costly membrane modifications were likely not compensated by a plasmid loss. Thus, the persistence of several large plasmids under high cellular stress conditions suggests that plasmid carriage does not inevitably reduce bacterial fitness (1, 102, 103).

Several pandemic high-risk clonal lineages have been identified in recent years, which successfully combine high-level virulence and resilience (1, 103–107) with extensive drug resistance, even in more pristine environments with low antibiotic selection pressures (108–110). This combination and the nonreversibility of resistance could be due to different compensatory events that counteract fitness costs (111). However, because of the broad phylogenetic diversity of bacterial hosts and the vast spectrum of compensatory mechanisms, reports and predictions of underlying adaptive processes that occur in clinical strains remain mostly unavailable. To address these issues, we performed a second EE analysis to track adaptational changes throughout the evolutionary process. We showed that the fitness costs of 2003.2 and 2003.9 were nearly completely ameliorated when evolved in two different media for 15 days (LB) and 25 days (MH-2). This rapid adaptation to external stress associated with fitness recovery might contribute to important changes in transmission dynamics and patient prognosis during infection (112). In addition, the fitness increase was accompanied by enhanced virulence levels of 2003.2^{LB}, $2003.2^{MH\text{-}2}$, 2003.9^{LB}, and $2003.9^{MH\text{-}2}$. While we identified mutations in *rpoE* and subsequent changes in expression levels of genes regulated by RpoE in the genomes of 2003.2^{LB} and $2003.2^{MH\text{-}2}$, our DGE analysis of 2003.9^{LB} and $2003.9^{MH\text{-}2}$ indicated the sole contribution of transcriptomic changes in the recovery of fitness and virulence. Note that the compensated fitness costs in the adapted variants 2003.2^{LB} and $2003.2^{MH\text{-}2}$ were accompanied by an increased susceptibility to colistin demonstrating collateral sensitivity, which might be a promising prospective therapeutic approach for treating chronic infections caused by XDR pathogens (49).

One limitation of our study is the "random" nature of mutations and transcriptomic and phenotypic changes, which might present differently upon repetition of the experiment. Moreover, during (experimental) evolution, numerous factors, such as variations in population size and selection pressure (113), might directly influence adaptational events. However, this does not diminish the significance of our findings but underlines the diversity of resistance evolution, compensation, and underlying processes. Another limitation is the possibility that mutations and changes in the mutants could be media adaptations that are difficult to correlate with natural circumstances (69). We cannot completely exclude this point without doubt. However, as can be seen in the DGE analysis, similar genes were up- or downregulated regardless of whether the second EE was performed in LB or MH-2 medium. The increased fitness and virulence of 2003.2^{LB}, $2003.2^{MH\text{-}2}$, 2003.9^{LB}, and $2003.9^{MH\text{-}2}$ was thus presumably not (only) based on media adaptations. Finally, to conclusively explain the underlying mechanisms behind the phenotypes would require depletion and/or complementation studies. Therefore, additional investigations will have to address these points further. Also, it might be promising to evaluate the impact of outer membrane modifications on the protective properties of promising vaccines whose immunization response is based on OmpK36 (114–116).

Conclusion. In this study, we not only highlight the important role of OmpK36 regarding β-lactam and AVI uptake as well as its contribution to resistance acquisition but also demonstrate the disadvantageous effects of changes in OMPs on bacterial fitness and virulence. More importantly, we show that fitness costs accompanied by this resistance acquisition were compensated rapidly and that bacterial virulence was almost completely restored, enabled by particular genomic and transcriptomic adaptations that involved major bacterial regulators.

MATERIALS AND METHODS

Bacterial strains and experimental evolution. Bacterial strains used in this study are listed in Table 1. All strains were stored at −80°C in LB (Carl Roth, Karlsruhe, Germany) supplemented with 20% (vol/vol) glycerol (anhydrous; Merck, Darmstadt, Germany). Before use, one single colony from fresh overnight cultures on LB agar plates was individually inoculated in 5 mL of LB and grown under shaking conditions (130 rpm) at 37°C overnight.

To study CAZ-AVI resistance acquisition and increase the probability of receiving resistant representatives (117), we used 12 randomly chosen single colonies as individual biological replicates of PBIO2003

TABLE 1 Overview of bacterial strains used in this study

Strain	ST	Relevant characteristics or genotype	References
Klebsiella pneumoniae			
PBIO2003	307	Wild-type strain (rectal swab, human), ancestral strain of 2003.2 and 2003.9 bla_{OXA-48}, $bla_{CTX-M-15}$, $bla_{SHV-106}$, bla_{TEM-1B}, *aac(3)-IIa*, *aph(3")-Ib*, *aph(6)-Ib*, *fosA*, *tet34*, *sul2*, *mdfA*, *oqxAB*	1, 13
2003.2	307	*ompK36* (Gln146*) mutated variant of PBIO2003 (first EE) Parental strain of 2003.2^{LB} and 2003.2^{MH-2}	This study
2003.2^{LB}	307	In LB-adapted variant of 2003.2 (second EE, 15 days)	This study
2003.2^{MH-2}	307	In MH-2-adapted variant of 2003.2 (second EE, 25 days)	This study
2003.9	307	*ompK36* (Δ78 bp; 972–1,049/1,125 nt) mutated variant of PBIO2003 (first EE) Parental strain of 2003.9^{LB} and 2003.9^{MH-2}	This study
2003.9^{LB}	307	In LB-adapted variant of 2003.9 (second EE, 15 days)	This study
2003.9^{MH-2}	307	In MH-2-adapted variant of 2003.9 (second EE, 25 days)	This study
ATCC 700603	489	Laboratory reference strain (urine, human), negative control for siderophore secretion assay	143
hvKP1	86	Archetypal hypervirulent *K. pneumoniae* isolate (blood and liver, human), positive control for hypermucoviscosity assay	27, 144
Escherichia coli			
PBIO1289 (IMT10740)	1159	Internal reference APEC strain (environment, poultry), positive control for serum resistance and survival in bile salts	108, 145
W3110	10	Laboratory reference strain, negative control for serum resistance and survival in bile salts	146

for our first EE approach (Fig. 1). The single colonies were inoculated in 1.5-mL tubes (Carl Roth, Karlsruhe, Germany) containing 1 mL of cation-adjusted Mueller-Hinton broth 2 (MH-2; Sigma-Aldrich, St. Louis, MO, USA) and grown at 37°C and 130 rpm. Following overnight incubation, 10 μL of bacterial precultures were transferred into a 96-well microtiter plate containing 190 μL MH-2 supplemented with CAZ-AVI (Zavicefta, Pfizer, New York City, NY, USA), resulting in a final concentration of 0.125/0.03mg/mL CAZ-AVI. The inoculated microtiter plates were incubated at 37°C without agitation for 24 h to allow "fixation" of mutations that occurred in the later growth phase (118). Daily, 10 μL of the culture was transferred in 190 μL of MH-2 with increasing concentrations of CAZ-AVI until some replicates tolerated concentrations of 16/4 μg/mL of CAZ-AVI. This is considered resistant according to the guidelines of EUCAST (42).

We then used a second EE approach to investigate compensatory events overcoming (putative) fitness burdens. One population, each of 2003.2 and 2003.9, was propagated in 5 mL of LB or MH-2 at 37°C and 130 rpm by transferring 5 μL of stationary-phase culture into fresh medium every day. We collected samples from the populations every fifth day and stored them at −80°C until further examination.

Whole-genome sequencing. One randomly chosen single colony was cultured overnight in MH-2 supplemented with 16/4 μg/mL CAZ-AVI. Total DNA was extracted using the MasterPure DNA purification kit for blood, version 2 (Lucigen, Middleton, WI, USA), according to the manufacturer's instructions. Isolated DNA was purity controlled and quantified using a NanoDrop 2000 and Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The DNA was shipped to the Microbial Genome Sequencing Center (MiGS; Pittsburgh, PA, USA) and, following library preparation, sequenced using 2 × 150 bp paired-end reads (Illumina NextSeq 550).

Raw sequencing reads of the parental strain PBIO2003 were processed as described before (1). The trimmed and filtered reads were mapped against the complete reference genome of the closely related strain PBIO1953 with breseq v.0.36.0 (119). Mutations evidenced by read alignments were applied to this reference with the help of the GenomeDiff tools shipped with breseq. This new reference of PBIO2003 was used for mapping sequencing reads of strains obtained through experimental evolution with breseq and deducing mutations.

Raw sequencing reads of evolved mutants were adapter-trimmed, contaminant-filtered, and quality-trimmed with BBDuk from BBTools v.38.89 (http://sourceforge.net/projects/bbmap/). FastQC v.0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for quality control of both raw and trimmed reads. *De novo* genome assemblies at a maximum coverage of 100× were performed using shovill v.1.1.0 (https://github.com/tseemann/shovill) with SPAdes v.3.15.0 (120). Draft genomes were additionally polished outside the shovill pipeline by mapping trimmed reads to the contigs of the draft assemblies using BWA v.0.7.17 (121), and after processing of SAM/BAM files (sorting, marking of duplicates) with SAMtools v.1.11 (122), variants were called with Pilon v.1.23 (123).

RNA isolation and sequencing. The overnight cultures were set to 0.5 McFarland standard turbidity. Then, 3 mL of these bacterial suspensions were added to 27 mL of LB (10-fold dilution) and incubated at 37°C and 130 rpm until the optical density at λ = 600 nm (OD_{600}) reached 0.2 turbidity (early log phase). Next, 1 mL of bacterial cultures was harvested, cooled down in liquid nitrogen for 5 s to inhibit further transcriptomic activity, and centrifuged (16,000 × *g* for 3 min at 2°C). Finally, the supernatants were discarded entirely, and the pellets were frozen in liquid nitrogen for 2 s and stored at −20°C (not more than 6 h) until further preparation. According to the manufacturer's instructions, the total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolated RNA was purity-controlled and quantified using a Qubit 4 fluorometer. The RNA was shipped frozen to LGC (LGC Genomics, Berlin,

Germany) and, following rRNA depletion and mRNA library preparation, sequenced using 1 × 75 bp reads (Illumina NextSeq 550, nonstranded).

First, Trim Galore v.0.6.7 (https://github.com/FelixKrueger/TrimGalore) was used for the adapter and quality trimming of the raw sequencing reads. Next, the trimmed reads were mapped with Bowtie 2 v.2.4.4 (124) (mode: --very-sensitive-local) using the assembly of PBIO2003 as reference. Subsequently, gene counts were calculated using featureCounts v.2.0.1 (125) based on the annotation of PBIO2003. Finally, the count table was imported into R v.4.1.1 (https://www.R-project.org/), and differentially expressed genes were called with DESeq2 v.1.33.5 (126) in default mode, with one exception; genes with rowSums of <10 in the count table were removed before the analysis. We used an absolute 1.5 $\log_2$ fold change threshold with an adjusted *P* value lower than 0.01 to determine differences in gene expression (127). We excluded one replicate of 2003.9^{LB} due to a shift on principal component 1 (PC1) and PC2 obtained by visual evaluation of the plotted principal-component analysis (PCA; Fig. S1 in the supplemental material). All differentially expressed genes were compared to the COG (Clusters of Orthologous Groups of proteins) database v.2020 (128, 129) using Diamond v.2.0.11.149 (130) (mode: blastP, E value of $\leq 10^5$).

Molecular modeling of OmpK36 protein structure. To investigate the molecular effects caused by mutations of *ompK36*, we predicted the protein structure of OmpK36 by homology modeling. The initial model for the trimeric full-length OmpK36 construct was built within the multiple sequence viewer application in Maestro v.2020-4 (Schrödinger, New York, NY, USA). In total, five models were calculated based on the template structure of Protein Data Bank (PDB) number 6RCP (54) using a knowledge-based approach with side chain optimizations. A high-quality, clash-free model was selected and subsequently prepared with the protein preparation wizard (131) in Maestro to add hydrogens, assign bond orders, and optimize protonation state, followed by a short restraint minimization. In order to obtain the structure for 2003.9, the residues Lys324 to Asp350 were simply deleted in all three monomers, and terminal residues were capped with acetyl groups at the N termini and protected *N*-methyl-amide at the C-terminal ends. CHARMM force field parameter and protein standard files (PSF) for NAMD v.2.13 (132) were prepared using CHARMM-GUI (133). Finally, hydrogen mass repartition was applied using an in-house script that enables a timestep of 4 fs (134). All stages were simulated *in vacuo* with a cutoff for nonbonded interactions at 1.6 nm, including a switching function with a 0.1-nm region. After initial minimization for 50,000 steps, the system was simulated for 4 ns NVT (constant number of particles, volume, and temperature) at 310 K. Temperature was controlled by a Langevin thermostat with a $1 ps^{-1}$ damping coefficient. The hydrogen bonds of β-sheet-forming residues were restrained using extra bonds with a force constant of 10.0 kcal/(mol × Å^2). Both ends of the open elliptical β-barrel were slowly contracted with harmonic potentials of 0.5 kcal/(mol × Å^2) in all three monomers simultaneously to close the region of segment deletion. Another trimeric homology was calculated from the 2003.9 sequence based on the final simulation snapshot as a template to rebuild the remaining loops with Prime (135).

Identification of outer membrane proteins by MALDI-TOF MS. The bacterial suspensions were set to 0.5 McFarland standard turbidity in deionized water, pelleted (13,800 × *g* for 5 min at room temperature [rt]), and resuspended in 300 μL of deionized water. Then, 900 μL of 99.8% (vol/vol) ethanol (Carl Roth, Karlsruhe, Germany) was added. Following two centrifugation steps (9,600 × *g* for 2 min at rt) and air drying (4 to 6 min), the pellets were resuspended in 20 μL of 70% (vol/vol) formic acid (Thermo Fisher Scientific, Waltham, MA, USA). The suspensions were mixed with 20 μL of acetonitrile (Carl Roth, Karlsruhe, Germany) and centrifuged (9,600 × *g* for 2 min at rt). Next, 1 μL of supernatants was spotted on a MALDI target plate (MBT Biotarget 96, Bruker Daltonik, Bremen, Germany) and left to dry at rt, and 1 μL of 130 μM 2,5-dihydroxybenzoic acid matrix (Sigma-Aldrich, St. Louis, MO, USA) was added onto each protein-containing spot. The extracted membrane proteins were analyzed by MALDI-TOF MS using a Microflex smart instrument (mass range of 2 to 40 kDa, laser intensity of 70%, number and frequency of shots of 200; Bruker Daltonik, Bremen, Germany). The analysis of spectra was performed using flexAnalysis software v.3.3 (Bruker Daltonik, Bremen, Germany).

Cellular uptake of CAZ, AVI, and ATM. The cellular uptake of the β-lactam antibiotics ATM (dissolved in water: dimethylformamide [DMF; 1:1]; Acros Organics, Geel, Belgium) and CAZ (dissolved in 0.1 M NaOH; Acros Organics, Geel, Belgium) and the non-β-lactam β-lactamase inhibitor AVI (dissolved in water; BioVision, Milpitas, CA, USA) was investigated by determining the increased cellular content of sulfur in terms of carbon monosulfide (λ = 258.0330 nm) based on uptake of the sulfurous compounds by using high-resolution continuum-source molecular absorption spectrometry (HR CS MAS) based on the graphite furnace technique. This investigation refers to experiments described previously (136) with minor modifications. First, overnight cultures were set to an OD_{600} of 0.3 turbidity (approximately 1.5 - × 10^8 CFU/mL). Then, 1 mL of these bacterial suspensions was transferred into 1.5-mL tubes and pelleted (4,000 × *g* for 10 min at rt). The supernatant was carefully discarded, and the pellets were resuspended in 1 mL of phosphate-buffered saline (PBS). Next, 100 μM sulfurous compound was added, and cells were incubated at 37°C without agitation. Samples containing only the solvent served as blanks. After 1 h of incubation, the cells were pelleted (4,000 × *g* for 10 min at rt) and washed once with 1 mL of PBS, and the collected pellets were stored at −20°C until further analysis.

For analysis, the pellets were thawed, resuspended in deionized water, and lysed by using a sonotrode (20 s, 9 cycles, 80 to 85% power; Bandelin Sonoplus, Berlin, Germany). An aliquot of the samples was spiked appropriately with the respective antibiotic and used for quantification. The measurements were performed with a contrAA 700 spectrometer (Analytik Jena, Jena, Germany). Ten microliters of the spiked samples was injected directly into pyrolytically coated graphite tubes (PIN-platform, Analytik Jena, Jena, Germany), followed by treatment applying for a time-temperature program as published before (137). Samples containing the lysate of bacteria were incubated only with the medium, and the

solvent served as a blank. Thus, the endogenous sulfur content (blank) was subtracted from the absorbances when investigating the uptake of the sulfurous compounds. The mean integrated absorbances of 2 to 3 injections were used throughout the experiments. The uptake was expressed as a percentage of relative reduction related to PBIO2003.

MIC. Phenotypic antimicrobial susceptibility testing (AST) was performed using the Vitek 2 automated system (bioMérieux, Marcy l'Etoile, France). The MIC values of CAZ-AVI, CAZ-AVI with ATM, tigecycline (dissolved in dimethyl sulfoxide [DMSO]; Acros Organics, Geel, Belgium), and chloramphenicol (dissolved in 99.8% [vol/vol] ethanol; VWR International, Radnor, PA, USA) were determined by broth microdilution according to ISO standard 20776-1. Additionally, the MIC of colistin was examined using MICRONAUT MIC-Strip colistin (Merlin Diagnostika, Bornheim, Germany) according to the manufacturer's instructions. All results were interpreted according to the published breakpoints and guidelines of EUCAST (42).

Growth kinetics and competition. Growth kinetics were assayed by continuously measuring the OD_{600}. Briefly, overnight cultures were diluted 1:100 in 5 mL of fresh LB and incubated at 37°C and 130 rpm until the OD_{600} reached 0.5 McFarland standard turbidity. Then, the bacterial suspensions were diluted 10-fold, and 200 μL of cultures was transferred in triplicates into a 96-well microtiter plate (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). Finally, the OD_{600} was recorded every 30 min using a microplate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany) at 37°C with 200 rpm orbital shaking.

The quantitative competition was determined by coincubation of PBIO2003 with 2003.2 and 2003.9 as well as 2003.2^{LB}, $2003.2^{MH\text{-}2}$, 2003.9^{LB}, and $2003.9^{MH\text{-}2}$. Here, the bacterial cultures were set to a 0.5 McFarland standard turbidity in LB as described for growth kinetics. Then, the reference (PBIO2003) and competitor strains were mixed in a 1:1 ratio by adding 100 μL of each strain to 20 mL of LB broth (approximately 1×10^5 CFU/mL each). Twenty microliters of the resulting suspension was used for serial dilutions on LB agar plates (total CFU/mL) and LB agar plates containing 16/4 μg/mL CAZ/AVI (CFU/mL of competitor strain) to receive inoculum sizes. Next, the inoculated suspension was incubated at 130 rpm and 37°C for 24 h. Finally, the CFU/mL of the strains was again determined by performing serial dilutions. The competitive indices (CIs) were calculated as the output/input ratio of the competitor compared to the reference strain.

Serum resistance and survival in bile salts. Determination of survival in 50% human serum and 50 mg/mL bile salts (an equal mixture of cholic acid and deoxycholic acid) was performed as described previously (138), with minor modifications. Briefly, overnight cultures were diluted 1:100 in 5 mL of fresh LB and incubated at 37°C and 130 rpm until the OD_{600} reached 0.5 McFarland standard turbidity. Then, bacteria were pelleted (7,500 $\times$ *g* for 5 min at rt) and resuspended in 1 mL of PBS. One hundred microliters of sample was seeded in a 96-well microtiter plate containing 100 μL of human serum (United States origin, Sigma-Aldrich, St. Louis, MO, USA) or bile salts (100 mg/mL, dissolved in PBS; Sigma-Aldrich, St. Louis, MO, USA) per well (resulting in a final concentration of 50% human serum or 50 mg/mL bile salts and approximately 1×10^8 CFU/mL). Next, 20 μL of each sample was collected, and the inoculum size was quantified by plating serial dilutions on LB agar plates incubated at 37°C overnight. The inoculated microtiter plates were incubated at 37°C without agitation for 4 h. After that, the number of survived CFU/mL was determined by plating serial dilutions and incubating at 37°C overnight. The positive control included in each experiment was the serum-resistant PBIO1289. The serum-sensitive W3110 served as the negative control. Serum resistance was expressed as $\log_2$ fold change of CFU/mL after treatment related to inoculum size. Percent survival in bile salts was obtained by determining differences in the CFU/mL after 4 h of incubation related to the inoculum count.

Siderophore secretion. The quantitative analysis of siderophore secretion was determined using a previously described method (24), with minor modifications. First, the bacterial cultures were set to 0.5 McFarland standard turbidity in 0.9% (wt/vol) NaCl solution. Then, 50 μL of bacterial suspension was added to 15-mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 5 mL of chelated M9 minimal salt medium (200 μM 2,2′-dipyridyl [Carl Roth, Karlsruhe, Germany] added M9 minimal salt medium [MP Biomedicals, Irvine, CA, USA]) supplemented with 2 mM $MgSO_4$ (Carl Roth, Karlsruhe, Germany) and 0.3 % (wt/vol) of Casamino Acids (c-M9-CA [139]; BD, Franklin Lakes, NJ, USA). The strains were grown for 24 h at 37°C and 130 rpm. Next, 1 mL of bacterial cultures was collected in 1.5-mL tubes and centrifuged (4,900 $\times$ *g* for 20 min at rt), and 100 μL of siderophore-containing supernatant was transferred in triplicates to 96-well microtiter plates containing 100 μL of CAS shuttle solution (composited according to ref. 140). Additionally, fresh medium (blank) and 15 mM EDTA (positive control; Carl Roth, Karlsruhe, Germany) were included. The nonsiderophore producer ATCC 700603 served as a negative control. The mixtures were incubated in the dark for 30 min at rt, and the absorbance at a λ = 630 nm was measured using a microplate reader (CLARIOstar Plus, BMG LABTECH, Ortenberg, Germany). Secretion of siderophores was expressed as siderophore production unit in percent calculated as published previously (141).

Hypermucoviscosity. The hypermucoviscosity sedimentation assay was performed as described previously (60), with the following modifications. Again, the bacterial cultures were set to 0.5 McFarland standard turbidity in 0.9% (wt/vol) NaCl solution, and 50 μL of these bacterial suspensions was added to 5 mL of LB. Following an incubation period of 24 h at 37°C and 130 rpm, 1.5 mL of the cultures was collected in 2.0-mL tubes (Carl Roth, Karlsruhe, Germany) and centrifuged (1,000 $\times$ *g* for 5 min at rt). Two hundred microliters of the upper supernatant and 200 μL of the incubated culture were separately transferred each into triplicates to 96-well microtiter plates, and the OD_{600} was measured. The mucoid phenotype was expressed as the ratio of supernatant to total OD_{600}.

Infection of *Galleria mellonella* larvae. The infections of larvae of the greater wax moth *G. mellonella* were performed as described previously (67). Briefly, 2 mL of overnight culture was collected and pelleted (16,000 × *g* for 5 min at rt). The pellets were washed once with PBS and diluted in PBS to an OD_{600} of 1.0 turbidity (approximately 2×10^9 CFU/mL). The bacterial suspensions were further diluted to 2×10^6 CFU/mL and 2×10^7 CFU/mL, respectively. The larvae (proinsects, Minden, Germany) were randomly divided into groups with 10 individuals in each group, and 10 μL of the bacterial suspensions was injected in the right proleg. Additionally, one group of larvae was injected with 10 μL of PBS to ensure that death was not due to trauma from the injection. Each group was placed in 90-mm glass petri dishes kept at 37°C in the dark, and death was recorded every 24 h. Individuals were considered dead when they did not respond to physical stimuli and showed pigmentation. The results obtained with three independent assays were pooled for each strain to generate Kaplan-Meier plots of mortality rates (142).

Statistical analysis. Statistical analyses were performed using GraphPad Prism v.9.3.0 for Windows (GraphPad Software, San Diego, CA, USA). All phenotypic experiments were performed with three or more independent biological replicates. Unless otherwise specified, data were expressed as mean and standard deviation. Assessment of statistical significance was performed via analysis of variation (ANOVA) with Dunnett's multiple comparison *post hoc* test. To analyze differences in cellular uptake of CAZ, AVI, and ATM, we used a one-sample *t* test. *P* values lower than 0.05 were used to show significant statistical differences among results.

Ethics approval. Ethical approval was given by the ethics committee of the University of Greifswald, Germany (BB 133/20). Informed patient consent was waived as samples were taken under a hospital surveillance framework for routine sampling. The research conformed to the principles of the Helsinki Declaration.

Data availability. The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB48690. Additional data of parental strains (PBIO1953 and PBIO2003) can be found under accession number PRJEB37933.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4 MB.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

E.E. and K.S. conceived and designed the study. E.E., J.A.B., C.L.C.-M., and D.B. performed the laboratory and phenotypic experiments. M.S., L.S., and S.E.H. performed the bioinformatics analyses. E.E., M.S., L.S., N.-O.H., J.A.B., U.T.B., S.E.H., J.U.M., F.A., K.B., C.L.C.-M., S.G., E.A.I., D.B., and K.S. analyzed the data. E.E. and K.S. wrote the manuscript with input from all co-authors, and E.E. prepared the tables and figures. All authors read and approved the final version of the manuscript.

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4 DISCUSSION

One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae* not only exhibit a range of MDR to PDR phenotypes, but also dominate as cause of infection in humans and animals [19]. It is therefore of utmost importance to determine the drivers and factors that contribute to their success, which is the major objective of this thesis.

4.1 One Health-related high-risk clonal lineages occur in rather neglected vectors and locations

The spread and dynamics of MDR *Enterobacterales* have been extensively studied worldwide. It is estimated that MDR *E. coli* and *K. pneumoniae* have reached exorbitantly high rates in the One Health dimensions of the Global South, which include a large number of LMICs [19]. However, the critical underpinnings of these assumptions remain elusive, largely because of the sparse and volatile data from these regions. In response, WHO, WOAH, FAO, and UNEP call for a more equitable approach to the various measures of the One Health approach between the Global North and Global South. Despite growing problems in sub-Saharan Africa regarding AMR, this region has not been studied as thoroughly as others. Thus, it is essential to investigate the factors that facilitate the spread and emergence of MDR *E. coli* and *K. pneumoniae* among humans, animals, and ecosystems in this region.

Sub-Saharan Africa is a region of Africa located south of the Sahara Desert that includes countries where the percentage of people living in poverty is the highest in the world [456]. In many parts, access to healthcare is severely limited due to lack of infrastructural and institutional capacities, especially in rural areas [457]. This lack of access to healthcare can lead to improper diagnosis and treatment of bacterial infections, resulting in overuse and misuse of antibiotics and increasing the likelihood of AMR development. Inadequate regulation and enforcement of antibiotic use exacerbate this matter, as uncontrolled over-the-counter sales of antibiotics are not uncommon [458]. For example, a study by Tuyishimire *et al*. (2019) found high levels of antibiotic self-medication among Rwandan students, particularly with the broad-spectrum β-lactam antibiotic amoxicillin, sometimes in combination with the β-lactamase-inhibiting clavulanic acid. The authors showed that students obtained antibiotics primarily from community pharmacies to treat symptoms unusual for bacterial infections, such as headache [82]. This example also highlights the communication barriers that contribute to the problem of AMR in sub-Saharan Africa. A survey conducted by the WHO showed that many people, including healthcare workers, were unfamiliar with the term AMR and that local languages often did not have an equivalent term or referred to it in an entirely different way (e.g., pain resistance) [459]. This unfamiliarity may contribute to the misconception that antibiotics are a panacea for most diseases, including symptoms of non-bacterial infections such as colds, fevers, or coughs. In addition, medical personnel are often unable to test for antimicrobial susceptibility, making it difficult to select an appropriate antibiotic [460]. As a result, broad-spectrum antibiotics are often prescribed empirically, contributing to antibiotic overuse and misuse [29, 80, 461–463].

Health facilities in sub-Saharan Africa face challenges in preventing the spread of bacterial pathogens due to limited resources or inadequate protocols. Faulty sterilization procedures for medical equipment and poor hygiene practices are common [460, 461]. Furthermore, lack of clean water, sanitation, and

waste disposal practices further exacerbate the problem [464–468]. All of these factors can lead to critical accumulation of various contaminants in the environment (e.g., antibiotic residues and heavy metals [467]), thereby promoting the selection and spread of resistant bacteria in soil, water, and air [465, 469]. In addition, the increasing conversion of natural habitats to agricultural land has brought humans into closer contact with animals, creating new opportunities for the transmission of zoonotic pathogens [469–471].

The “perfect storm” theory suggests that a number of factors in sub-Saharan Africa, such as poor sanitation, unreliable water supply, inadequate waste disposal, close human-animal contact, including livestock in urban residential areas, and the extensive use of broad-spectrum antimicrobials not only in medicine but also in agriculture, may favor the spread of resistant pathogens [469, 470]. Studies have also found a high prevalence of MDR *E. coli* and *K. pneumoniae* in wildlife [267, 472–474], including frugivorous (*Eidolon helvum*) and insectivorous bats (*Nycteris hispida*) [475], rats (*Rattus rattus*, *R. norvegicus*, and *Cricetomys gambianus*) [267], cattle egrets (*Bubulcus ibis*) [476], and nonstinging insects such as the housefly (*Musca domestica*) [477–479]. The latter is a ubiquitous insect typically found in livestock production and related facilities such as slaughterhouses, where larvae can develop in feces and organic material [480]. Although thought to have originated in the steppes of Central Asia, they are now distributed worldwide and are found in rural and urban areas in tropical or temperate climates [481, 482]. Houseflies can become contaminated through contact with surfaces, waste, or water and can transmit pathogens through their exoskeleton and alimentary canal [483, 484]. Therefore, houseflies are not only underestimated reservoirs for a variety of microorganisms [485, 486], but also suitable sentinels for continuous monitoring of the prevalence of clonal lineages and spatial distribution of AMR [487]. Indeed, there is evidence that houseflies can indicate changes in the prevalence of ESBL-producing *E. coli* in urban and rural areas [488]. Their unrestricted movement gives them the unique ability to access hard-to-reach areas and can thus reveal the presence of AMR in different niches [489]. To date, there is a large number of published studies describing the carriage of MDR *Enterobacterales* by flies, including *Citrobacter* spp., *Enterobacter* spp., *E. coli*, and *Klebsiella* spp. In addition, enteropathogenic pathogens, such as *Salmonella enterica* (*S. enterica*) and *Yersinia enterocolitica*, as well as Gram-positive pathogens, including *Bacillus anthracis*, *Enterococcus* spp., and (methicillin-resistant) *Staphylococcus aureus*, have also been frequently detected (e.g., [483, 490–499]). *Musca domestica* has also been found contaminated with fungi, parasites, and viruses [481].

In **Publication 1**, we drew attention to the role of houseflies as mechanical vectors of MDR high-risk clonal lineages of *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacterales* with highly virulent genotypes, including the One Health-related high-risk *E. coli* lineages ST131 and ST410 in an African setting [113]. While ticks and mosquitoes are commonly known as vectors for pathogens such as *Borrelia burgdorferi* and *Plasmodium* spp., which are associated with Lyme disease and malaria, respectively [500, 501], houseflies tend to be a neglected vector, particularly in hospitals in sub-Saharan Africa [481]. Our findings show that houseflies are reservoirs for MDR bacterial pathogens, some of which are known to cause severe infection in humans and domestic animals [113]. However, due to the unrestricted movement and undemanding lifestyle of houseflies, predicting their occurrence, as well as controlling and eliminating them, is challenging [480]. Therefore, it is critical to consider rather neglected

vectors in the context of One Health to develop a comprehensive approach for controlling the spread of AMR that takes into account the interrelationships among human, animal, and environmental health [502].

Despite the One Health-related high-risk clones ST131 and ST410, we identified other known *E. coli* lineages such as ST167 and ST617 in the sample set of houseflies [113]. In their recent review, Kocsis *et al*. (2022) showed that ST167 and ST617 are epidemiologically successful high-risk clonal lineages associated with AMR. Interestingly, both lineages are single-locus (ST167; *purA*) or double-locus (ST617; *purA*, *recA*) variants of ST10, a typical commensal *E. coli* clonal lineage belonging to phylogroup A [204]. Compared to ST10, *E. coli* ST167 and ST617 have numerous allelic variations related to anaerobic metabolism, such as the propionate kinase TdcD, which is actively involved in the conversion of ADP to ATP in absence of oxygen, or the reactivase EutA, which activates the ethanolamine ammonia-lyase that allows ethanolamine to be used as a nitrogen and carbon source under anaerobic conditions [150, 503]. Similar mechanisms are frequently reported in other pathogenic species of *Enterobacterales* that cause severe disease in mammals (including humans), such as *Yersinia* spp. and *Salmonella* spp. [504–506]. Evidence suggests that these variations observed in anaerobic respiration allow pathogens to outcompete benign gut bacteria, providing a fitness advantage in successfully establishing and colonizing new hosts [507]. In addition to enhanced competitive growth, the anaerobic metabolism genes of ST167 and ST617 allow the use of oxidized sulfur compounds as electron acceptors, which are increasingly released by the host immune system response at the site of inflammation. Furthermore, there is evidence of lineage-specific alterations in intergenic regions of ST167 and ST617 [503], a phenomenon previously described as a cause of the emergence of MDR plasmid-containing *E. coli* ST131 strains [199]. All of these allelic variations appear to be key events in the transition from a commensal to a broad host range pathogen. To date, MDR (including carbapenemase-producing) ST167 and ST617 clones have been reported worldwide as causing severe infection in humans and animals (e.g., [503, 508–512]) and have also been detected in various wildlife and environmental sources (e.g., [513–516]).

The presence of ST131, ST167, ST410, and ST617 in our sample set of houseflies provides a compelling argument for the prevalence of (putative) One Health-related high-risk clones in sub-Saharan Africa. As noted above, there is generally weak evidence on the exact extent of AMR in sub-Saharan Africa and the prevalence of high-risk clones with a One Health background. To investigate the latter further in addition to houseflies, we took a snapshot of the presence of ESBL-producing *E. coli* that occurred in and around the University Teaching Hospital of Butare, Rwanda, during the sampling period from October to December 2014. In **Publication 2**, we examined 120 of these bacteria from hospitalized patients, their caregivers, family members, neighbors, and companion animals. Our study revealed low genetic diversity, as 70.8% (85/120) of the strains belonged to eight predominant STs, including the aforementioned (potential) One Health-related high-risk lineages ST131, ST648, ST410, ST617, and ST167 (sorted by decreasing prevalence) [112]. In addition, we found that ST38, ST354, and ST405 were among the predominant phylogenetic backgrounds in our setting, all known as causative agents of bacterial infection in humans and animals (e.g., [133, 517–521]). These results support the hypothesis that One Health-related high-risk clonal lineages are prevalent in locations of sub-Saharan Africa.

Plasmids and plasmid conjugation are critical factors in the spread and stabilization of AMR especially in *Enterobacterales* [118, 522–527]. During conjugation, the donor and recipient cell must establish a mating pair and maintain contact long enough for successful DNA transfer [302]. This process appears to be favored in the mammalian gastrointestinal tract [528–531], where biofilms on the intestinal mucosa promote the transfer of IncF plasmids [532–534]. However, the transfer of other plasmid replicon types is thought to be inhibited in the presence of mammalian cells, possibly due to host factors affecting plasmid transfer rates [535]. Interestingly, transfer rates appear to be higher in insects, suggesting that nonvertebrate reservoirs, such as houseflies, may be important hotspots for the exchange of resistance plasmids among different *E. coli* and *K. pneumoniae* lineages [536, 537].

To investigate plasmid contents in different *E. coli* lineages in sub-Saharan Africa, we analyzed plasmid sequences in-depth for **Publication 2**, revealing not only ST-specific similar plasmids, but also a high degree of similarity among those from isolates with different STs including from humans and houseflies [112]. This suggests that the spread of AMR in sub-Saharan Africa is driven not only by the circulation of One Health-related high-risk clonal lineages, but also by the transmission of resistance plasmids.

Overall, while further research is needed to understand the full extent of AMR in sub-Saharan Africa, the results of this thesis contribute to the understanding of the occurrence and transmission dynamics of One Health-related high-risk clonal lineages in this area. However, it is important to keep in mind that the sample collection took place back in 2014 and that the distribution of STs and AMR phenotypes in the African setting could be different in the interim. Yet, it can be inferred from these data that One Health-related high-risk clonal lineages were numerically the most abundant in our sample set, a trend that is also confirmed in more recent studies (e.g., [140, 194–198]).

4.2 One of the drivers of successful clonal lineages: Combination of antimicrobial resistance and virulence

The global human population is expected to reach 8.79 billion by the end of the century [538], posing significant challenges to ensuring that people have access to safe, healthy, and nutritious food. To meet the increasing demand for food, global food production needs to increase by more than 50% of 2012 production levels [539]. However, from a One Health perspective, food, including vegetables and meat, can also be a source of pathogens that cause food-borne infectious diseases [540–542]. Rearing, harvesting and slaughtering, processing, preparation, and consumption of food can therefore have an impact on human, animal, and environmental health.

Food-borne pathogens can cause a variety of diseases in humans and animals, especially livestock, ranging from gastroenteritis to life-threatening conditions such as sepsis [540]. Moreover, these pathogens can contaminate soil, water, and other natural sources, ultimately affecting the health of plants, wildlife, and ecosystems [45, 543–546]. Therefore, it is critical to take a holistic view at food and implement effective food safety measures, such as proper food handling and preparation, regular monitoring and testing of food products, and appropriate waste management, to identify contaminants and prevent the spread of food-related pathogens and mitigate their impact on human, animal, and environmental health [547]. Accordingly, it is imperative that hazardous food-borne pathogens can be reliably identified and potential transmission routes are interrupted at an early stage.

Traditionally, food-borne infections related to pathogenic *E. coli* have been associated with those affecting the gastrointestinal tract and caused by InPEC, such as the hybrid InPEC that emerged in Northwestern Germany in 2011 [548]. However, a growing body of research, such as the one conducted by Pietsch *et al*. (2018), has shown that food can also be an important reservoir for antimicrobial-resistant One Health-related high-risk ExPEC lineages [549]. Given their ability to colonize the host after ingestion [137], food can be a driving force for the spread of AMR at the human-animal interface and a source of pathogens that cause life-threatening bacterial infections. However, the pathogenic source of these infections can be difficult to unequivocally identify, as infections may occur long after the pathogen has colonized the intestine [550]. As a result, reports of food-borne extraintestinal infections, such as recently defined food-borne urinary tract infections, are rare (e.g., [551–553]).

With the advent of high-throughput DNA sequencing, comparative genomics has largely replaced traditional phenotypic analysis as the basis for understanding natural selection and bacterial virulence. However, the phenotype of a pathogen is what actually causes disease through the expression of VFs and is fought by the host immune system. Therefore, to predict bacterial virulence and pathogenic potential in the absence of a clinical event (e.g., when isolating a pathogen from food), genotypic markers must be linked to virulent phenotypes. In this regard, Mitchell *et al*. (2015) established genotypic and *in vitro* phenotypic criteria characteristic of each pathotype in the ExPEC group, allowing assignment of 282 *E. coli* strains from chicken meat and eggs to a specific pathotype. However, the authors noted that the classification of pathotypes failed in some cases, possibly misinterpreting the proportion of certain pathotypes [398]. Despite efforts to identify a genetic marker set that predicts a particular pathogenic outcome, such as uropathogenesis, this has not yet to be achieved [554]. This underscores the uniqueness of virulence enabled by specific VFs, likely determined by a combination of factors and specific to the pathogen under study (possibly also depending on its evolutionary past).

In **Publication 3**, we developed/adapted a set of *in vitro* and *in vivo* virulence phenotyping methods that allows pathogen characterization according to clinical aspects, including biofilm formation, siderophore secretion, resistance to complement-mediated killing, and the ability to cause mortality in *G. mellonella* larvae. By combining phenotypic results with genomics, our study provided a comprehensive characterization of a food-borne MDR *E. coli* strain [120].

The *E. coli* strain PBIO3502 studied in **Publication 3** was isolated from a fresh pork sausage bought in Germany. Genomic analysis revealed that it belonged to ST58, an ExPEC lineage and single-locus variant of ST155 (*gyrB*). *E. coli* ST58 is abundant in all One Health dimensions worldwide (e.g., [198, 549, 555, 556]) and it meets all criteria for being defined as an One Health-related high-risk clonal lineage [557]. Despite its ubiquity in surveys, ST58 is often overlooked compared to better-known clonal lineages such as ST131 and ST410.

While ST58 belongs to phylogroup B1, which is typically considered less virulent, it is essential to note that infections caused by ST58 strains are severe (e.g., [549, 555, 556, 558, 559]). Therefore, the assumption that phylogroup B1 bacteria are generally less virulent should be reconsidered. Further evidence for this contradiction is provided by our genomic analysis of PBIO3502, which revealed that it carried over 145 virulence-associated genes, including those required for biofilm formation, serum

resistance, and siderophore secretion. These features were evaluated phenotypically, confirming a highly virulent phenotype and pathogenic potential, which was also associated with high motility rates in the *G. mellonella* larvae infection model [120].

For a long time, MDR bacteria were thought to gain a selection advantage at the expense of lower fitness and virulence, at least immediately after acquiring resistance [214]. It has also been suggested that ubiquitous opportunistic pathogens such as *E. coli* and *K. pneumoniae* can colonize and exhibit high levels of virulence only in specific hosts, a phenomenon known as host specificity [560–562]. However, recent evidence suggests that One Health-related high-risk clonal lineages exhibit both "generalism" and "phenotypic plasticity" with respect to their virulence. Generalism refers to the ability of these lineages to use the same mechanisms in different niches to survive, persist, and outcompete other phyla [199]. Phenotypic plasticity, on the other hand, describes their ability to modify the expression of virulence and fitness features in response to changing environmental conditions [447, 563–566].

Bacterial virulence is complex and appears to be the result of natural selection [567]. While selection for resistance properties relates to the presence of antibiotics or other co-selective agents such as heavy metals or biocides in the microbiosphere, the selection process for virulence is less well understood. It is possible that expression of VFs does not necessarily correlate positively with pathogenic success of high-risk clonal lineages in specific hosts in terms of host damage and mortality, which would reduce the chances of transmission. Instead, VFs could be a byproduct of selection for adaptations primarily unrelated to disease, and their maintenance provides advantages in a variety of habitats both within and outside a host (e.g., the anaerobic respiration of ST167 and ST617). Therefore, it is also plausible that some bacterial pathogens only coincidentally infect mammalian hosts (humans and/or animals) [568], and that some of their VFs originally evolved to target non-mammalian organisms such as bacterivorous protozoa and nematodes, predatory bacteria and fungi, or phages (e.g., [569–574]). For example, *E. coli* develops a mucoid phenotype, exhibits increased fitness, filamentation, and shows distinct ability to form biofilms when co-cultured with predatory myxobacteria [570], characteristics known to be prerequisites for uropathogenesis [438, 439, 441, 575]. Conversely, *E. coli* attenuates the virulence of the parasitic amoeba *Entamoeba histolytica* [576, 577], the causative agent of amebiasis in humans and animals. Given that One Health-related high-risk clonal lineages inhabit diverse environmental niches and have a broad host range, including wildlife, it is likely that they have evolved a variety of survival strategies based on the acquisition and maintenance of distinctive traits that potentially enhance their ability to cause disease.

In summary, the results from this thesis regarding virulence significantly contribute to the assessment of the clinical relevance/pathogenicity potential of strains that were not directly derived from a clinical infection, e.g., from food. Moreover, they not only highlight the importance of the combination of AMR and bacterial virulence for the successful emergence of One Health-related high-risk clonal lineages, but also show that this combination may be the "Achilles' heel" that could be prospectively utilized. Exploiting their virulence properties could be one of the future aspects of alternative antibacterial therapies [578, 579]. In this regard, the recently approved sideromycin cefiderocol, which exploits the bacterial expression of siderophore receptors [580], is a good example.

4.3 Another driver of successful clonal lineages: Compensatory mechanisms

In **Publication 4**, we used two successive serial transfer experiments to study resistance development against the fixed drug combination CAZ-AVI and subsequent reaction to the acquired resistance mechanism in terms of fitness and virulence. In the first approach, we increased the selection pressure daily by increasing concentrations of CAZ-AVI and obtained two resistant variants (2003.2 and 2003.9) within 14 days. We then removed the selection pressure and incubated the mutants in two different growth media until the adapted variants showed an increase in their growth characteristics. We therefore determined growth every fifth day using growth kinetics, which showed a significant increase in fitness compared to the resistant variants after 15 and 25 days, respectively [119].

Assuming a generation time of 18–20 minutes (based on the results of the German *K. pneumoniae* ST307 outbreak clone [118]) and an expected exponential growth of five hours, both resistant mutants emerged during the first EE approach in **Publication 4** after approximately 1,300 bacterial generations, which is consistent with previous studies (e.g., [391, 581–583]). In the absence of antibiotic selection pressure, mutation rates of proteobacterial pathogens are approximately 10^{-10} to 10^{-11} mutations per genome per generation [584–587]. However, increasing selection pressure can increase this mutation rate up to 10^{-5} mutations per genome per generation [365, 588], with mutations leading to genomic rearrangements (such as insertions, deletions, and duplications) being the most common [589–591]. It seems likely that the effects of such genomic rearrangements are very rarely, if ever, counterbalanced by reverse mutations and therefore play a crucial role in the evolution and stabilization of AMR [591, 592].

Our genomic analysis revealed that mutations in *ompK36* were responsible for resistance to CAZ-AVI [119]. Although these mutations are not artificial and are common in clinical *K. pneumoniae* strains (e.g., [41, 364, 367]), alterations in membrane permeability have not been adequately linked to decreased uptake of the first approved non-β-lactam β-lactamase inhibitor avibactam [593]. However, we proved this beyond doubt by cellular uptake studies, as the intracellular concentration of a β-lactamase inhibitor determines its efficacy [594]. Interestingly, both types of mutations appear to be equally effective in terms of their contribution to CAZ-AVI resistance, which is consistent with the results of previous studies (e.g., [364, 595, 596]). Acosta-Guitérrez *et al*. (2018) showed that in the case of reduced pore diameter, the electrostatic potential and the electric field within the pore increase, hindering the permeation of the mainly anionic or zwitterionic β-lactams, while allowing nutrients such as glycine to pass [597]. In turn, the reduced expression or absence of OmpK36 in the outer membrane restricts the transit of β-lactams across the outer membrane, preventing them from reaching their target in the periplasmic space [367, 598].

In addition to a premature stop codon in *ompK36*, our genomic analysis uncovered a read-through mutation in *dsbA* in 2003.2, which may affect protein expression and stability [599]. DsbA is a thiol oxidase responsible for the proper folding and stability of periplasmic proteins, including OmpA [600] and MCR [601]. Many bacterial extracytoplasmic proteins depend on the formation of disulfide bonds between cysteine residues for their stability [602]. A recent study by Furniss *et al*. (2022) demonstrated that inhibition of DsbA activity sensitizes MDR *E. coli* and *K. pneumoniae* strains to several antibiotics, including ceftazidime [601]. However, our study did not confirm these findings, as the resistance profile

of 2003.2 compared to wild-type PBIO2003 differed only in resistance to CAZ-AVI [119]. Thus, our findings support the notion that mutations in *ompK36* are responsible for resistance to β-lactams and β-lactamase inhibitors [595, 596].

There is evidence that reduction of membrane permeability in *K. pneumoniae* is usually accompained by a reduction in fitness and virulence characteristics [363, 366]. However, the study by Fajarod-Lubián *et al*. (2019) suggests that the extent of reduction in fitness and virulence differs between mutation types [596]. Using our large panel of *in vitro* and *in vivo* assays compiled in **Publication 3**, we confirmed and demonstrated differences in the reduction of fitness and virulence-associated features depending on the mutation type of *ompK36*. The most significant burden was observed with complete loss of OmpK36 (2003.2), whereas a reduction in pore diameter (2003.9) resulted in a smaller reduction in fitness and *in vitro* virulence compared with wild-type. Interestingly, the reduction in *in vivo* mortality in *G. mellonella* larvae was comparable in both mutants [119]. This may suggest that other factors or mechanisms influence the pathogenic effect of *K. pneumoniae* in the host. Therefore, it is conceivable that the two resistant variants have different VFs or expression profiles that influence the results in the *in vitro* assays but not necessarily in the *G. mellonella* larvae. This highlights the context-dependence of bacterial virulence and that pathogen characterization by individual *in vitro* assays is not sufficient to determine the true virulence of *K. pneumoniae*. While *in vitro* assays can be a useful tool to characterize the basic properties of bacteria, they cannot fully mimic the complex interaction between the pathogen and the host.

In contrast to the development of resistance, our second EE approach showed that fitness recovery did not occur until between 1,600 and 2,700 generations after the removal of selection pressure [119]. Indeed, compensatory mutations, which can restore fitness by mitigating the negative effects of resistance, seem to occur more slowly than the development of resistance itself [376]. Consequently, numerous studies measuring the growth of bacteria that have recently acquired resistance compared to their susceptible counterparts in laboratory environments without selection pressure have shown that pathogens undergo fitness trade-offs that compromise their pathogenic potential in order to maintain resistance, potentially then leading to reverse mutations (e.g., [316, 318, 368]). However, this is likely due to the lack of competitive pressure in standardized laboratory environments and may be difficult to reproduce experimentally [603]. Nevertheless, evidence suggests that the genetic basis for resistance development, including incorporation of mobile genetic elements or chromosomal mutations, associated costs, and genomic location (i.e., chromosome or plasmid), are critical factors in the success of compensatory mutations [340, 604–608].

In **Publication 4**, genomics and transcriptomics combined with *in vitro* and *in vivo* phenotyping revealed that not only transcriptomic changes were the reason for the significant increase in fitness and overall virulence, but also compensatory mutations in *rpoE* of the essential envelope stress response system (so-called σ^E pathway) [119]. RpoE is activated in response to extracytoplasmic stressors such as heat shock or misfolded outer membrane proteins [609, 610]. Once activated, RpoE binds to the bacterial core RNA polymerase complex (RNAP) to form a holoenzyme that regulates the expression of approximately 100 genes [611, 612]. These genes include the small non-coding RNAs (sRNAs) such as MicA and RybB, which inhibit the expression of major outer membrane proteins to reduce the

accumulation of misfolded porins within the periplasm [613, 614]. Along with other members of the RpoE regulon, these sRNAs control the expression of outer membrane proteins and other bacterial envelope components to maintain or restore envelope integrity [615–617]. Simultaneously, the RpoE-RNAP holoenzyme induces transcription of several proteins associated with a delay in binary fission and thus overall growth, including proteins required for DNA recombination and repair [611, 618].

Previous studies examining the fitness and virulence of different bacterial species reached conflicting conclusions about the role of RpoE or other stress response components in these traits. For example, Huang *et al.* (2019) showed that downregulation of *rpoE* attenuated the virulence of *Pseudomonas plecoglossicida*, including reduced adhesion and biofilm formation [619]. Studies by Humphreys *et al*. (1999), Osborne and Coombes (2006), and Zhang *et al.* (2016) proved that RpoE promotes invasion and intracellular survival by regulating the expression of a type III secretion system of *S. enterica* and *Salmonella typhimurium* (*S. typhimurium*), respectively [620–622]. Recently, Thoda *et al.* (2023) demonstrated that subinhibitory concentrations of rifampicin, which inhibits activity of RNAP, suppressed the production of capsular polysaccharides in hvKp, resulting in decreased mucoviscosity [623]. Moreover, RpoE-regulated genes have been described to be important for urinary tract colonization by uropathogenic *E. coli* [624]. In contrast, Xue *et al.* (2015) showed that induction of the σ^E pathway by zinc ions attenuated virulence of InPEC [625]. Shetty *et al.* (2019) found that virulence-associated features, including biofilm formation under fluent conditions and the ability to swarm, were unaffected in an *S. enterica* Δ*rpoE* mutant compared to the wild-type strain [626]. Moreover, knocking out *rpoE* in Gram-positive *Streptococcus mutans* increased not only virulence but also fitness [627]. Presumably, these bimodal results are attributable to unphysiologically high or low levels of guanosine tetraphosphate ppGpp in response to the overexpression or knockout approaches of the respective studies [628]. In summary, further research is needed to validate the role of RpoE and the envelope stress response in restoring fitness and virulence-associated features in MDR bacteria.

Our study suggests that loss of RpoE function in *K. pneumoniae* ST307 can lead to an increase in both fitness and virulence-associated features. However, this increase was “purchased” by a loss of resistance to colistin [119]. Colistin resistance in the *K. pneumoniae* ST307 outbreak clone depended on the activity of the PhoPQ two-component system, which is positively regulated by RpoE. Therefore, loss of RpoE function resulted in an increase in anionic binding sites on the lipid A of the LPS for the cationic polypeptide colistin [356–359]. Accordingly, several reports have shown that downregulation of PhoPQ restores colistin susceptibility in MDR *K. pneumoniae* [358, 629–634]. Similar mechanisms of colistin sensitization have been demonstrated in other Gram-negative pathogens, including *Enterobacter cloacae*, *E. coli*, *S. enterica*, *S. typhimurium*, and *Vibrio cholerae* [360, 620, 635–638].

In its strictest interpretation, collateral sensitivity refers to the phenomenon that resistance to one antibiotic leads to increased susceptibility to another antibiotic [639]. However, our study has shown that collateral sensitivity may have a broader meaning, encompassing an increase in susceptibility to one antibiotic through compensatory mutations that offset the fitness and virulence costs associated with acquiring resistance to another antibiotic, particularly in combination therapy [119]. Recent research has focused on studying the occurrence and mechanisms of collateral sensitivity as a reliable and sustainable therapeutic strategy, but most studies have been limited to laboratory strains that may not

have been exposed to antibiotics in their evolutionary past and to a few bacterial species with a limited number of phylogenetic backgrounds (e.g., [640–646]). Moreover, there are few studies that have examined whether collateral effects are consistent across different clinically relevant resistance alleles and strains to the same antibiotics (e.g., [647–651]). This is particularly noteworthy because the results of our and other studies suggest that resistance to a given antibiotic may arise from mutations in different genes or at different locations within a gene, leading to different phenotypic outcomes such as changes in minimum inhibitory concentration and bacterial fitness or virulence (e.g., [119, 375, 651–653]). Interestingly, certain resistance plasmids can also have collateral effects on their bacterial host [654].

Despite many efforts, there is currently no therapeutic regimen that employs collateral sensitivity in clinical practice. As pointed out in [13], there are practical challenges in implementing therapeutic strategies based on collateral sensitivity. First, it can be difficult to identify generalizable treatment strategies for a variety of pathogenic strains, as the antibiotic pairs that trigger collateral sensitivity may differ even among phylogenetic backgrounds of the same species [655, 656]. Second, different resistance mutations can lead to either collateral sensitivity or cross-resistance, which may provide pathogens with a way to circumvent the desired effect of switching between antibiotics [648, 657]. Third, the rate at which a pathogen regains sensitivity is often much slower than the rate at which it develops resistance [119, 642, 658]. Finally, the sequence in which drugs are administered may be critical in certain circumstances because evolution is irreversible [659].

Taken together, compensatory mechanisms appear to be one of the major biological drivers for the successful propagation and stabilization of AMR, particularly in One Health-related high-risk clonal lineages. These lineages appear to have been exposed to a variety of selective influences throughout their evolutionary history within different One Health dimensions [660]. A deeper understanding of the mechanisms underlying compensatory evolution may open new avenues to exploit the vulnerabilities of these harmful pathogens. Moreover, extending the idea of collateral sensitivity and applying it to One Health-related high-risk clonal lineages could lead to more sustainable therapeutic strategies. One promising approach might be to anticipate the evolutionary potential of pathogens and direct them to evolutionary dead ends [650].

However, a limitation of this study is that it focused on examining the compensatory mechanisms of a limited number of *K. pneumoniae* ST307 mutants. While the results provide valuable insight into the potential mechanisms underlying resistance development and mitigation of accompanying fitness costs, it is important to recognize that the scope of the study was limited to a single strain. Therefore, further studies are needed to validate and extend these findings. Conducting larger studies with multiple strains and different genetic backgrounds would provide a more comprehensive understanding of the compensatory mechanisms in One Health-related high-risk clonal lineages and improve the generalizability of the results.

4.4 Dual-use research of concern in the One Health context

The dual-use dilemma is a complex issue that arises in the field of life sciences and other disciplines. It occurs when scientific research has the potential to be used for both benevolent and malicious purposes [661]. This may include research on the development of AMR, pathogenic agents such as filoviruses, arenaviruses, *Burkholderia mallei*, and *Yersinia pestis*, or toxins that could potentially be used to develop biological weapons or to intentionally harm humans, animals, and/or the environment. Although researchers have the constitutional right to intellectual inquiry and freedom of research, they must be aware of the risks associated with the potential misuse of their research findings [662]. The dilemma for researchers therefore arises from the possible actions of others, including extortion for financial gain, biowarfare, and bioterrorism.

The threat of bioterrorism is a real concern, as demonstrated by the Amerithrax bioterrorist attack that occurred one week after the September 11 terrorist attack in the USA. In this bioterrorist attack, spores of *Bacillus anthracis* were sent via the U.S. Postal Service [663], resulting in the death of 5 people and infection of 17 others [664].

Advances in genetic engineering have made it possible to enhance the virulence, transmissibility, or AMR of naturally occurring pathogens and to produce and weaponize appropriate biological agents. However, it can be argued that studies demonstrating simple ways to identify and promote high-level of virulence and AMR in bacteria (e.g., **Publication 3** and **4**) should not be conducted or published, as this could alert bioterrorists to new opportunities to produce biological weapons and provide explicit instructions for doing so [665].

Data and information sharing has a long tradition in the life sciences. However, the potential dual-use risks associated with scientific research in the life sciences led the U.S. government to establish the National Science Advisory Board for Biosecurity (NSABB). In 2007, the NSABB defined seven categories of research of concern because of their dual-use potential and sought to guide researchers and policymakers in evaluating the potential risks and benefits of their work. The goal was to prevent the development of biological agents or toxins that could be used for malicious purposes. One such category is improving the resistance and virulence properties of a biological agent [666], which certainly applies to the adapted variants in **Publication 4** [119].

It is important to note that the presence of dual-use potential in research does not automatically mean that it should not be conducted or shared. It does mean, however, that the conduct and disclosure of such research should be carefully considered from the outset and throughout the research process. This responsible approach is necessary to avoid potential risks and to ensure that research is conducted in a safe and ethical manner [666]. Therefore, our decision to conduct and disclose our research responsibly is justified. By critically weighing the potential risks and benefits, we have ensured that our work has been conducted with appropriate safeguards, while minimizing the potential for harm and promoting scientific progress.

Managing the risks associated with dual-use research of concern requires careful balancing between promoting scientific and technological progress and protecting the public from potential harm. However, dual-use research is a complex issue that raises ethical and safety concerns, and it is an area where

the One Health approach can be particularly valuable in addressing these concerns and finding solutions that protect the health and welfare of all stakeholders. In doing so, the One Health approach could address dual-use research in several ways: (i) through collaboration and open communication among different disciplines, One Health can help ensure that the potential risks and benefits of dual-use research are carefully weighed from different perspectives; (ii) by developing guidelines and protocols for conducting dual-use research responsibly and ethically, and by establishing mechanisms for monitoring and review, ensure that such research is conducted safely and ethically; and (iii) through education and training help researchers understand the potential risks and ethical considerations associated with dual-use research and help them identify ways to mitigate those risks [667].

5 CONCLUSION AND OUTLOOK

Antibiotics were once a powerful tool of modern medicine, but the increasing development of AMR in dangerous bacterial pathogens poses a major challenge to human and veterinary medicine. One Health, which advocates interdisciplinary and cross-sectoral collaboration to address complex health issues at the human-animal-ecosystem interface, is a promising approach to this global burden. However, while anthropogenic perturbations (e.g., overuse and misuse of antibiotics) have undoubtedly influenced natural selection for bacteria with AMR determinants such as MDR *E. coli* and *K. pneumoniae*, we still know little about why certain high-risk clonal lineages of these pathogens thrive in diverse global environments and a variety of human and animal hosts, while others are restricted to specific habitats and hosts. This thesis contributes to our understanding of the drivers and factors that determine the international success of One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae*.

First, particular successful high-risk clonal lineages drive the spread of AMR and contribute greatly to the stabilization of AMR genes in a variety of bacterial niches across the One Health dimensions. These clonal lineages have adapted to survive and persist in a wide variety of hosts and environments, making them globally prevalent. It appears that they are widespread not only in well-studied regions of the Global North, but also in less-studied areas of the Global South.

Second, One Health-related high-risk clonal lineages seemingly combine multidrug resistance with high bacterial virulence. This dangerous combination makes them particularly concerning, as they can cause recalcitrant and difficult-to-treat infections even in otherwise healthy hosts.

Finally, after acquiring resistance, One Health-related high-risk clonal lineages may initially experience a loss of fitness and virulence, including reduced growth, capsule formation, and increased mortality in *G. mellonella* larvae infection models. However, it appears that these effects can be reversed after a short time, while AMR remains stabilized. Compensatory mechanisms may be one of the most important factors in the success of One Health-related high-risk clonal lineages, as they allow them to acquire and maintain multiple AMR determinants and stabilize AMR within bacterial populations. In addition, the rapid restoration of fitness and virulence makes One Health-related high-risk clonal lineages highly competitive in any niche of the various One Health dimensions, including infection sites.

In summary, this thesis provides valuable insights into the genetic and functional traits that contribute to and drive the successful dissemination of One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae* in and across diverse global environments and niches. By examining genetic, phenotypic, and evolutionary mechanisms, this research improves our understanding of the fundamental principles that determine epidemiological success. Ultimately, this work contributes to the growing body of knowledge about the diverse interfaces of One Health and provides an initial foundation for the development of novel and sustainable therapeutic strategies to combat AMR.

Moving forward, interdisciplinary collaboration and data sharing will be critical to addressing global health challenges. Given the increasing global spread of AMR and the pivotal role that One Health-related high-risk clonal lineages play in its distribution, it is crucial to implement effective surveillance and prevention measures, particularly in the understudied regions of the Global South. Progress in this area will require not only accurate approximations, but also the development of novel

methodological approaches to generate standardized data that will ultimately facilitate the implementation of effective surveillance and prevention strategies. A major challenge will be to deepen our understanding of the dynamics of One Health-related high-risk clonal lineages of *E. coli* and *K. pneumoniae* to prevent transmission within and across One Health dimensions. To address this challenge, wildlife barriers and other innovative prevention strategies will be invaluable.

Apart from developing prevention strategies, there is a growing need for novel therapeutic approaches that target MDR bacteria without harming the healthy microbiome. But how can we combat AMR and harness the pathogenic potential of One Health-related high-risk clonal lineages for treatment? One promising approach might be to identify fitness- and virulence-associated features and use antifitness or antivirulence strategies. Rather than relying on forced transport of antibacterial agents such as cefiderocol, one could shift towards “disarming” the pathogen, enabling the host immune system or competing commensal bacteria to eliminate the pathogen itself. However, an essential aspect is defining success indicators for these novel therapeutic strategies. In contrast to evaluating antibiotic efficacy by determining the minimum inhibitory concentration, evaluating the efficacy of antivirulence strategies can be more challenging. Therefore, it is necessary to define diagnostic success markers to effectively evaluate the efficacy of these therapeutic strategies. The panel of *in vitro* and *in vivo* typing methods developed in this thesis could be used and further refined to meet these specific requirements.

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APPENDIX

A1 Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Ort, Datum	Elias Eger

A2 Curriculum vitae

Remark: The curriculum vitae has been removed from the electronic version of this dissertation.

A3 Publications and scientific achievements

Publications

2023 Schurig S., Kobialka R., Wende A., Khan M.A.A., Lübcke P., **Eger E.**, Schaufler K., Daugschies A., Truyen U., and Wahed A.A.E. Rapid reverse purification DNA extraction approaches to identify microbial pathogens in wastewater. *Microorganisms*. 2023;11(3):813. doi: 10.3390/ microorganisms11030813

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Wolff L., Murthy S., **Eger E.**, Lam H.-N., Napierkowski M., Baecker D., Schulzke C., and Bednarski P. Comprehensive evaluation of biological effects of pentathiepins on various human cancer cell lines and insights into their mode of action. *International Journal of Molecular Sciences*. 2021;22(14):7631. doi: 10.3390/ijms22147631

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Heiden S.E., Kurz M.S.E., Bohnert J.A., Bayingana C., Ndoli J.M., Sendegeya A., Gahutu J.B., **Eger E.**, Mockenhaupt F., and Schaufler K. Flies from a tertiary hospital in Rwanda carry multidrug-resistant Gram-negative pathogens including extended-spectrum β-lactamase-producing *Escherichia coli* sequence type 131. *Antimicrobial Resistance and Infection Control*. 2020;9(1):34. doi: 10.1186/s13756-020-0696-y

Conference contributions

Oral presentations **Eger E.**, Schwabe M., Schulig L., Hübner N.-O., Bohnert J.A., Bornscheuer U.T., Heidecke C.-D., Kramer A., Balau V., Gierer W., Schaefer S., Eckmanns T., Gatermann S.G. Heiden S.E., Müller J.U., Adnan F., Becker K., Correa-Martínez C.L., Guenther S., Idelevich E.A., Baecker D., and Schaufler K. In-depth analysis of a convergent *Klebsiella pneumoniae* ST307 outbreak clone from Germany and how it counters the fitness and virulence costs associated with acquired resistance. Junior Scientist Symposium 2022. November 2022: Friedrich Loeffler-Institute, Island Riems-Greifswald (Germany).

Eger E., Schwabe M., Schulig L., Hübner N.-O., Bohnert J.A., Bornscheuer U.T., Heiden S.E., Müller J.U., Adnan F., Becker K., Correa-Martínez C.L., Guenther S., Idelevich E.A., Baecker D., and Schaufler K. Extensively drug-resistant *Klebsiella pneumoniae* rapidly compensates for fitness and virulence costs associated with acquired resistance. 74. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) e.V. September 2022: DGHM, Berlin (Germany)

Eger E., Heiden S.E., Korolew K., Bayingana C., Ndoli J.M., Sendegeya A., Gahutu J.B., Kurz M.S.E., Mockenhaupt F., Müller J., Simm S., and Schaufler K. Phylogenetic characterization of multidrug-resistant *Escherichia coli* from patients, the community, livestock and flies in Rwanda. One Health Conference 2022. April 2022: Helmholtz Institute for One Health, Greifswald (Germany)

Poster presentations **Eger E.**, Schwabe M., Maschkowitz G., Schubert S., Bueter A., Franzenburg S., Fickenscher H., Krumbholz A., and Schaufler K. Diversity of virulence properties of *Burkholderia cepacia* complex isolates that caused non-respiratory tract infections. 33rd European Congress of Clinical Microbiology and Infectious Diseases 2023. April 2023: European Society of Clinical Microbiology and Infectious Diseases, Copenhagen (Denmark)

Poster presentations (continued)

Heiden S.E., Kurz M.S.E., Bohnert J.A., Bayingana C., Ndoli J.M., Sendegeya A., Gahutu J.B., **Eger E.**, Mockenhaupt F., and Schaufler K. Multidrug-resistant Gram-negative pathogens carried by flies from a tertiary hospital in Rwanda. Tagung der Deutschen Veterinärmedizinischen Gesellschaft (DVG) e.V. Fachgruppe “Bakteriologie und Mykologie”. June 2021: online

[Heiden S.E., Kurz M.S.E., Bohnert J.A., Bayingana C., Ndoli J.M., Sendegeya A., Gahutu J.B., **Eger E.**, Mockenhaupt F., and Schaufler K. Multidrug-resistant Gram-negative pathogens carried by flies from a tertiary hospital in Rwanda. 72. Jahrestagung der DGHM. March 2020: cancelled]

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