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Mechanisms and evolution of colistin resistance in clinical Enterobacteriaceae

Mechanismen en evolutie van colistin resistentie in klinische Enterobacteriaceae

(met een samenvatting in het Nederlands)

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1 General introduction

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Escherichia coli and *Klebsiella pneumoniae* as multidrug-resistant Gram-negative opportunistic pathogens

Escherichia coli and *Klebsiella pneumoniae* are facultative anaerobic, rod-shaped, Gram-negative opportunistic pathogens in the Enterobacteriaceae family, and are asymptomatic colonizers of the intestinal microbiota (1–3). In addition, *K. pneumoniae* also asymptotically colonizes the skin, and the upper respiratory tract. *E. coli* and *K. pneumoniae* are frequently observed pathogens, that can cause infections outside of their respective niches. Common opportunistic infections caused by both *E. coli* and *K. pneumoniae* are bloodstream, and urinary tract infections (4–7). Individually, *E. coli* is well known to cause enteric infections (1, 5), whilst *K. pneumoniae* may also cause soft tissue infections, and pneumonia (2, 8). *K. pneumoniae*, together with *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and species from the *Enterobacter* genus, is a member of the group of ESKAPE pathogens (9). Together, like the acronym of their genus names suggests, they are known for their ability to evade the effects of antibiotic treatments in patients, through various mechanisms of antibiotic resistance. Due to the increasing rate of antibiotic resistance, it has been suggested to include *E. coli* in this group; forming the ESKAPEE pathogens (10).

The rate of antibiotic resistance in both *E. coli* and *K. pneumoniae* has increased all over the world, over the last two decades (11, 12). Both species have become increasingly resistant to multiple types of antibiotics, including fluoroquinolones, third-generation cephalosporins, and aminoglycosides (5, 11, 13, 14). Through the spread of carbapenemases located on mobile genetic elements, *K. pneumoniae* has also increasingly acquired carbapenem resistance (5, 11, 13–16). Some countries report sporadic outbreaks of carbapenem-resistant *K. pneumoniae*, but other countries suffer from endemic presence these types of resistant *K. pneumoniae* (5, 16). Thus, the prevalence of carbapenem resistance in *K. pneumoniae* varies greatly between countries. Within the European Union, the rate of carbapenem resistance in invasive *K. pneumoniae* isolates has been determined to vary between 0% and 64% between countries. Within carbapenem-resistant *K. pneumoniae*, the rate of resistance against colistin in carbapenem-resistant *K. pneumoniae* has been estimated at 28% (17). In contrast to *K. pneumoniae*, the rate of carbapenem resistance in invasive *E. coli* isolates is lower, with 0-2% of isolates reported as resistant, depending on the individual country (13, 18).

A notable point in the increasing number of infections caused by multidrug-resistant *E. coli* and *K. pneumoniae* is the spread of particularly multidrug-resistant lineages. Within *E. coli*, the ST131 lineage is known for its ability to cause extra-intestinal infections (1). Within the ST131 lineage, the C clade

is particularly well-known for its multidrug-resistant nature, with the acquisition of CTX-M class extended-spectrum ß-lactamases, making this group resistant to third-generation cephalosporins. Furthermore, clade C ST131 *E. coli* strains have also acquired fluoroquinolone resistance through point mutations in DNA gyrase and DNA topoisomerase genes (19, 20). Within *K. pneumoniae*, the ST15, ST147, ST258, and ST395 are well known multidrug-resistant lineages. These lineages can harbour CTX-M class extended-spectrum ß-lactamases, but also NDM-type, or OXA-48-type carbapenemases (21–23). Some of these multidrug-resistant *E. coli* and *K. pneumoniae* lineages are also particularly well-equipped to cause infections through the acquisition of virulence factors on the mobile genetic elements that can encode the antibiotic resistance genes (24).

The increasing rate of multidrug resistance, including carbapenem resistance in Enterobacteriaceae and other types of Gram-negative bacteria, has led to a revival in the use of colistin as a last-resort drug to treat infections with these opportunistic pathogens (25). In some countries, failures in hospital hygiene measures has lead an endemic spread of carbapenem-resistant *K. pneumoniae* lineages. In these countries, this spread has led to an particular increase in the use of colistin in order to treat infections with these pathogens (26, 27). However, as the use of colistin has increased, outbreaks with carbapenem-resistant, but also colistin-resistant *K. pneumoniae* in clinical settings are increasingly frequent (23, 28–31). The increasing prevalence of Enterobacteriaceae that are resistant to both carbapenems, and colistin, is of growing concern for public health.

Colistin: a Gram-negative selective antibiotic

Colistin belongs to the polymyxin class of antibiotics. Polymyxin antibiotics were first isolated from *Paenibacillus polymyxa* in 1947 and were first used in clinical settings in the 1950s (32). Polymyxins are amphipathic, non-ribosomally synthesized, cyclic lipopeptides, composed of a decapeptide with an intramolecular heptapeptide loop, and an N-terminal fatty acid group (Figure 1A). A number of different polymyxins have been described, varying in their exact composition of amino acids in the peptide portion, and fatty acid group (32). From the ten amino acids, those in positions 3, 6, 7, and 10 may vary. The fatty acid group may vary from a seven- to a nine-carbon fatty acid, and may be methylated, hydroxylated, or sulphonated (32–34).

Although there is variation in the exact composition of the each polymyxin, the amphipathic structure of polymyxins is conserved. The hydrophobic aspects of polymyxins is conserved in both the fatty acid group, and the hydrophobic

characteristics of the variable amino acids in position 6 and 7 (Figure 1A) (32, 34). The hydrophilic nature is conserved in the polar residue of threonine at position 2, the cationic residues of the L-α,γ-diaminobutyric acid (Dab) groups in positions 1, 5, 8, and 9, and the conserved hydrophilic characteristics of the variable amino acids in positions 3 and 10 (32, 34). These hydrophobic and hydrophilic traits of the polymyxin molecule allow the molecule to fold into an amphipathic structure. This characteristic is crucial to the mechanism of action of polymyxins. Since colistin is the most widely used polymyxin, and because all polymyxins have similar characteristics, we have used colistin as a representative for the polymyxin class of antibiotics in the rest of this thesis (32, 33).

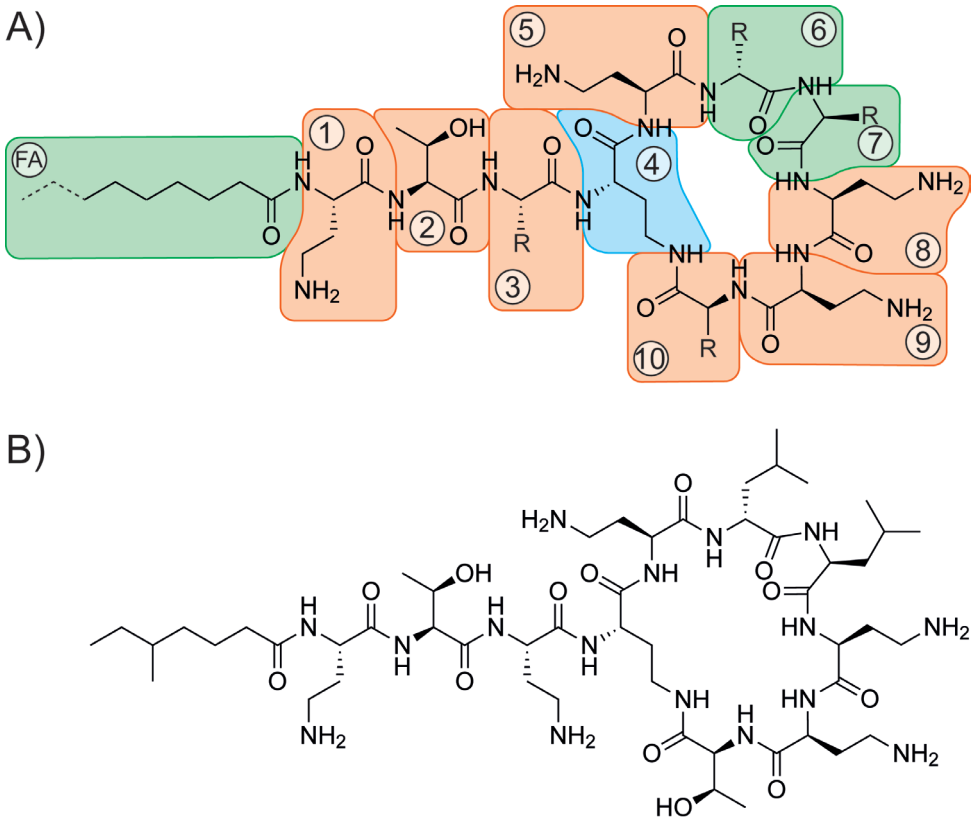

Figure 1: Chemical structure of polymyxins and colistin. A) General chemical structure of polymyxins. Polymyxins are composed of a non-ribosomally synthesized decapaptide (amino acids are number 1 through 10) with an intramolecular loop (amino acids 4 through 10), linked to a fatty acid (FA) (32). The fatty acid may differ between a seven- and nine-carbon fatty acid, and may be methylated, hydroxylated, or sulphonated. The variable residues of the peptide portion of polymyxins (residues of amino acids 3, 6, 7, and 10) are denoted with an "R". The individual amino acids, and the fatty acid, are coloured according to their function within the molecule. Hydrophobic moieties have a green background, hydrophilic moieties have an orange background. The L-Dab amino acid in position 4 coloured with a blue background, functions as the linker to form the intramolecular loop. **B)** Chemical structure of colistin B (polymyxin E2). All forms of polymyxin E carry L-Dab in position 3, D-Leu in position 6, L-Leu in position 7, and L-Thr in position 10. Colistin B has a 6-methylheptaoyl fatty acid chain (32).

Colistin is the type of polymyxin in which the variable groups are: L-Dab at position 3, D-leucine at position 6, L-leucine at position 7, and L-threonine at position 10 respectively (Figure 1B) (32, 35). Colistin comes in two different forms, in which the fatty acid group varies between (S)-6-methyloctanoyl (polymyxin E1, or colistin A), and 6-methylheptanoyl (polymyxin E2, or colistin B) (32, 35). Commercial preparations of colistin consists of a mixture of both colistin A and colistin B (36). Although these forms differ in their fatty acid group, differences in their bactericidal activity are negligible (37).

Colistin is selectively bactericidal for Gram-negative aerobic bacilli, and does not affect Gram-positive bacteria, and acid-fast bacteria. Colistin is only active against Gram-negative bacteria because of the presence of LPS molecules in the membranes of this type of bacteria (32, 38–40). Some specific Gram-negative aerobic bacilli are however, intrinsically resistant to colistin. These species include: *Brucella* spp., species from the *Burkholderia cepacia* complex, *B. pseudomallei*, *Edwardsiella tarda*, *Hafnia* spp., *Helicobacter pylori*, *Morganella morganii*, *Ochrobactrum intermedium*, *Proteus* spp., *Providencia* spp., *Serratia* spp., species from the Sphingomonadaceae family, and *Vibrio* spp. (32, 33, 41–44). These species mediate intrinsic resistance by the constitutive modification of the phosphate groups of lipid A with cationic molecules, to diminish anionic charges, or by reduction of the number of phosphate groups (45). In addition, colistin is not active against Gram-negative cocci, and anerobes.

The LPS molecules found in the membranes of Gram-negative bacteria are vital for colistin, since colistin relies on the interaction with LPS molecules for its mechanism of action (32, 38–40). The mechanism of action of colistin starts when colistin comes into close proximity of the Gram-negative bacterium (Figure 2, step 1). Colistin can outcompete the divalent Ca^{2+} and Mg^{2+} cations that are normally bound to the anionic phosphate groups of these lipid A moieties, and bind to the lipid A portion of LPS molecules through electrostatic interactions (Figure 2, step 2) (46). After binding to lipid A, colistin can insert itself into the membrane as a result of the conserved amphipathic structure (Figure 2, step 3) (46, 47). Although the initial insertion of colistin into the outer leaflet of the outer membrane leads to the weakening of membrane integrity, destabilization of this membrane does not lead to cell death (48). Because of observations that outer membrane destabilization did not lead to cell death, the exact mechanism through which colistin kill Gram-negative bacteria had long been debated (49, 50). Recent work has shown that colistin migrates through the periplasmic space (Figure 2, step 4), and will target the LPS molecules that are located in the outer leaflet of the inner membrane after synthesis in the cytoplasm (Figure 2, step 5) (51). The destabilizing effect of colistin on the inner membrane, leads to the death

of the bacterium (Figure 2, step 6) (39, 51).

Besides the use of colistin to treat infections with multidrug-resistant Gram-negative bacteria, colistin is used as a prophylactic drug in a treatment known as the selective decontamination of the digestive tract (SDD). SDD is used to efficiently lower the 28-day mortality and morbidity in patients admitted in an intensive care unit (ICU) (52, 53). In these critically ill patients, infections are an important cause of morbidity, and mortality, with more than half of ICU-admitted patients developing an infection during their stay (54). These infections are often caused by Gram-negative bacteria, and Enterobacteriaceae in particular. These often originate from the endogenous gut microbiota of the patient (54–56). The SDD treatment is designed to clear these bacteria from the patients gut. SDD consists of the oral administration of a suspension of three non-absorbable antimicrobials: the antibiotics tobramycin, and colistin, and the antifungal amphotericin B; and the intravenous administration of a biliary-excreted third-generation cephalosporin during the first four days of ICU admission. The combination of these drugs eradicates the gut of yeasts, *Staphylococcus aureus*, and aerobic Gram-negative bacteria, whilst leaving the anaerobic portion of the microbiota intact, ensuring colonisation resistance (53). However, the success of SDD in lowering the infection rate, and 28-day mortality, has only been observed in countries with low antibiotic resistance rate (57). Furthermore, the use of SDD

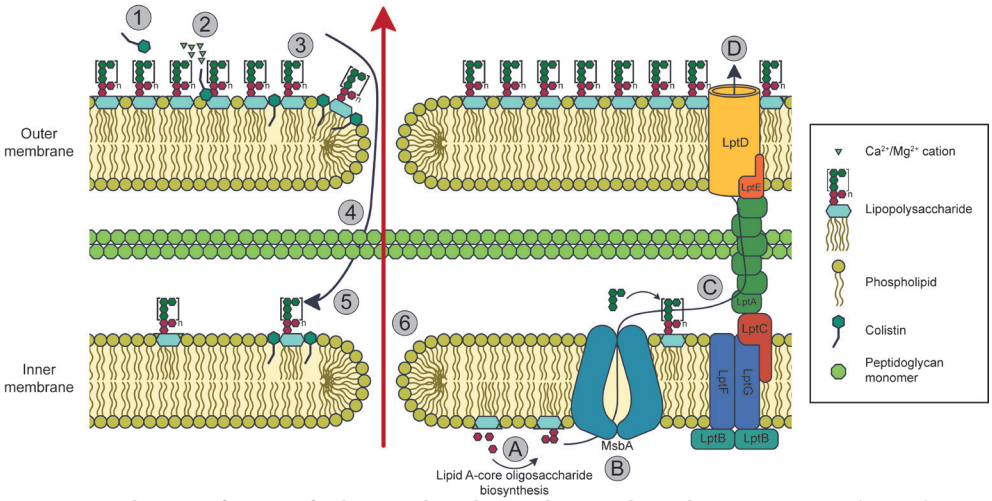

Figure 2: Mechanism of action of colistin and LPS biosynthesis and membrane transport. The mechanism of action relies on colistin coming into close proximity of the Gram-negative bacterium (**step 1**), after which the colistin molecule may bind to the anionic phosphate groups of lipid A (**step 2**). After insertion, and destabilization of the outer membrane (**step 3**), colistin can migrate further into the cell (**step 4**), and may bind the lipid A molecule present in the inner membrane (**step 5**), after which the destabilization of the inner membrane kills the Gram-negative bacterium (**step 6**). LPS is present in the outer leaflet of the inner membrane after the biosynthesis of lipid A-core oligosaccharide the cytoplasm (**step A**), and transport by MsbA (**step B**). LPS will be transported to the outer membrane after extraction by the $LptB_2FG$ ABC transporter complex, and transport by LptC and LptA (**step C**). Translocation to the outer leaflet of the outer membrane occurs through the LptDE complex (**step D**).

has been linked to outbreaks of multidrug-resistant Gram-negative bacteria, resistant to aminoglycosides, third-generation cephalosporins, and colistin. Also, observations on the overall increase of the rate of resistance within these pathogens within the ICU have been reported (58, 59). The use of SDD, and thus colistin, in these settings, has become increasingly controversial (60).

Outside of human medicine, colistin is an effective drug in used in veterinary settings. The veterinary use of colistin is mainly found in animal husbandry, in the use as growth promoter, and to treat enteric infections. In these settings, colistin can be used to treat an large number of animals by mixing colstinsulphate in the food provided (61). As colistin has a last-resort status in human medicine, the (aspecific) use in animal husbandry, has been criticized (61). In addition, the veterinary use of colistin has been linked to the spread of colistin-resistant organisms, some of which harbour plasmids encoding mobile colistine resistance (*mcr*)-genes, to the environment (62, 63).

Because of its cationic charge, colistin is a non-absorbable drug. Colistin is thus used to efficiently treat topical infections like otitis and skin infections (38). However, due to the toxic side effects of colistin, parental administration is ill-advised. Instead, the pro-drug version of colistin, colistimethate sodium (CMS), in which the Dab-residues have been sulphomethylated, may be used when parental administration is necessary (32). The sulphomethylation changes the cationic charge of colistin to an anionic one, but also renders the compound inactive (38). Hydrolysis of the sulphomethyl-groups will return the molecule to the active, free base, form (64). Within this thesis, we have not used CMS for any experimentation and thus we will not further discuss CMS.

As mentioned, besides potent bactericidal effects, colistin also has toxic side-effects. These effects include nephrotoxic side-effects, but also neurotoxic side-effects (e.g. dizziness, weakness, and neuromuscular blockade), respiratory toxicity, gastrointestinal disorders, and effects the skin including skin rash, itching, urticaria, and hyperpigmentation (32). Of these, the nephrotoxic side-effects are observed most prevalent (32, 64–66). The nephrotoxic effects stem from the renal filtration and subsequent reabsorption within a nephron (32). After aspecific filtration of the plasma fraction of blood in the glomerulus, colistin is actively reabsorbed in the proximal tubulus of the nephron by epithelial cells (32, 67, 68). Colistin is efficient reabsorbed through the peptide transporters 1 and 2 (PEPT1/PEPT2), the carnitine/organic cation transporters 1 and 2 (OCTN1/OCTN2), or endocytosed after binding to the megalin receptor (32, 67–69). The high expression of these reabsorption mediators leads to the accumulation within the epithelial cells.

This accumulation of colistin is hypothesized to have necrotic effects on the epithelial cells through the same amphipathic nature that is crucial for its bactericidal effects. The amphipathic nature results in a detergent-like effect on cellular membranes. The detergent-like capabilities are hypothesized to increase the permeability of these membranes, leading to increased influx of water and ions, and resulting in the necrosis of the cells (67, 70, 71). Colistin may also affect the integrity of intracellular structures, like mitochondria and the endoplasmatic reticulum, leading to the loss-of-function of these organelles (67, 72). In addition, colistin has been described to lead to cell cycle arrest, oxidative stress, and activation of apoptotic pathways, damaging the epithelia of the nephron in the process (32, 67–69).

To prevent toxic side-effects, optimizations to parental colistin (or CMS)-containing treatments are actively being sought. Since the toxic side-effects are dose-, and duration-dependent, optimization of the dosing-strategy may limit these effects (32, 66, 73, 74). In addition, parallel treatment of patients with compounds that compete for binding the reabsorption transporters, may reduce the nephrotoxic side-effects (75, 76). Other interventions to reduce toxicity may include the increased hydration of patients, to influence the concentration of colistin in the filtrate of the nephron (77). Lastly, the influence of the pH of the filtrate in the nephron has been suggested to have an effect on colistin-reabsorption through reduction of the cationic charges (76, 77).

Biosynthesis and assembly of lipopolysaccharides

The LPS molecules targeted by colistin are highly abundant in the outer leaflet of the outer membrane of Gram-negative bacteria and have a vital role in the structural integrity of the outer membrane, making them essential for Gram-negative bacteria (78, 79). LPS molecules are composed of three distinct parts: lipid A, the core oligosaccharide, and the variable O-antigen. The lipid A portion of LPS is composed of a phosphorylated disaccharide, which is acylated with multiple fatty acid groups (80). The amphipathic lipid A is membrane anchored, and will bind divalent cations (e.g. Ca^{2+} or Mg^{2+}) to the phosphate groups, establishing a highly impermeable barrier in the outer leaflet of the outer membrane (78, 80). The core oligosaccharide of the LPS molecule is composed of several monosaccharide groups, and may include non-carbohydrate groups such as phosphate groups (51). The core oligosaccharide links lipid A and O-antigen. The O-antigen is the surface-located part of the molecule, and is composed of a variable number of repeats of a variable group of saccharide molecules (79). The O-antigen may be variably present in the LPS synthesized in Gram-negative bacteria. If present, the

LPS is considered smooth, LPS without the O-antigen is termed rough (78, 81).

The biosynthesis of LPS starts within the cytoplasm of the Gram-negative bacteria by the biosynthesis of lipid A (81). The lipid A biosynthesizing Lpx-protein family synthesizes keto-deoxyoctulosonate $(KDO)_2$-lipid A from uridine diphosphate (UDP)-N-acetylglucosamine (GlcNac) (81). Although LpxA catalyzes the first step within this synthesis, the deacetylation of UDP-3-O-acyl-GlcNAc by the highly regulated LpxC dictates the first committed step in KDO_2-lipid A synthesis (82). After synthesis of KDO_2-lipid A, the addition of the core oligosaccharide is catalysed by glycosylstransferases, resulting in lipid A-core oligosaccharide (Figure 2, step A) (51, 81, 83). This product however, is still situated in the inner leaflet of the inner membrane. To transport the lipid A-core oligosaccharide to the outer leaflet of the inner membrane, the lipid A-core oligosaccharide ATP-binding cassette transporter MsbA binds and transports this molecule across the membrane (Figure 2, step B) (51, 81, 83). As discussed before, it is here that colistin may act in its bactericidal capacity, through destabilization of the inner membrane (Figure 2, step 5, step 6) (39).

When located in the outer leaflet of the inner membrane, the lipid A-core oligosaccharide may be glycosylated with the O-antigen through one of three independent O-antigen synthesizing pathways that accumulate in the transfer of the synthesized O-antigen to the lipid A-core oligosaccharide by the WaaL O-antigen ligase (51, 80). From the outer leaflet of the inner membrane, the LPS molecule, with or without O-antigen is transported to the outer leaflet of the outer membrane through the trans-envelope machinery formed by the Lpt-protein family (51, 83). The LPS molecule is extracted from the inner membrane by the $LptB_2FG$ ABC transporter complex (51, 83). The LptC protein can than bind the LPS, and transfer it to the multiple copies of LptA that span the gap to the LptDE complex in the outer membrane (Figure 2, step C). LptD serves as the ß-barrel shaped pore in the outer membrane through which the LPS molecule is passed, whilst LptE serves as a chaperone to LptD in this process (Figure 2, step D) (51, 80, 83).

LPS, and thus the genes that encode the proteins that act in the biosynthesis and assembly of LPS, are generally considered essential in Gram-negative bacteria. Some species that defy this dogma have been observed. *A. baumannii*, *Chlamydia trachomatis*, *M. catarrhalis*, and *Neisseria meningitides*, have been observed to be able to lose their LPS. As a result however, these species may experience a reduced fitness. LPS deficient *A. baumannii*, *N. meningitidis*, *M. catarrhalis* will have a decreased virulence upon infection (84–86). In addition, *A. baumannii* will experience an increased susceptibility to the human antimicrobial peptide LL-37 when LPS is lost (84). *C. trachomatis* will lose its ability to infect new

individuals without LPS (83). Some species of Gram-negative bacteria are always devoid of LPS. These species are *Sphingomonas paucimobilis*, *Borrelia burgdorferi*, and *Treponema pallidum* (83). All the species that (variably) lack LPS in their membranes however, compensate for the absence of LPS through the increased synthesis in glycolipids, and lipoproteins, in addition to increased presence of the normal membrane phospholipids constituents in the outer leaflet of the outer membrane (79, 83, 87).

Mechanisms of colistin resistance

The increasingly vital role of colistin as a last-resort drug, has increased the interest into the possible mechanisms of resistance that can develop. As mentioned, the mechanism of action of colistin consists of two distinct steps; first, the electrostatic binding of the colistin molecule to lipid A, and subsequently the insertion of its hydrophobic domains into the membrane (Figure 2). Both these steps occur at the two distinct membranes of Gram-negative bacteria (39). By interrupting one of these steps, bacteria may render themselves resistant to colistin. Thus far, several distinct mechanisms of acquired resistance have been described (Figure 3).

The most frequently observed mechanism of colistin resistance is the modification of the phosphate groups of lipid A by addition of a cationic molecule. The addition of cationic molecules to the anionic phosphate groups diminishes their net anionic charge. The reduction of anionic charge reduces the possible electrostatic interactions between colistin and lipid A. The cationic molecules that may be used are: 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (PEtN), or galactoseamine (GalN) (45, 88). The addition of L-Ara4N is performed by the *arn*-operon, encoding ArnBCADTEF and Ugd (together also known as PmrHFIJKLME) proteins that facilitate the biosynthesis and addition of L-Ara4N to the phosphate group of lipid A. PEtN is added to lipid A by the PEtN transferase PmrC (EptA in *E. coli*), from phosphatidylethanolamine (89). The proteins responsible for the addition of GalN to lipid A are homologs of *Francisella* FlmF2 and FlmK (88, 90). However, the modification of lipid A with GalN is less extensively described than the modification with L-Ara4N, or PEtN. The modification of lipid A with cationic molecules has been described in a large number of nosocomial Gram-negative bacteria. The modification with L-Ara4N is frequently observed in nosocomial strains of *K. pneumoniae* (91–93), and *P. aeruginosa* (94) strains. The addition of PEtN is observed in *A. baumannii* (95), *E. coli* (96), and strains carrying an *mcr* gene (97). GalN addition so far has only been observed in an colistin-resistant strain of *A. baumannii* (88).

A less frequently observed mechanism to modify the anionic charges carried by lipid A is the loss of one of the phosphate groups of lipid A through the actions of LpxE. The loss of one of the phosphate groups of lipid A has been described in *Salmonella enterica* subsp. *enterica* serovar Typhumurium (98), *A. baumannii* (95), *Rhizobium* species (99), and *Francisella* species (100), but not in *Escherichia* and *Klebsiella* species.

Outside the modification of the anionic charges of lipid A, LPS may be modified in several ways to develop resistance. The modification of the acyl-groups through the addition of an hydroxyl-group, forming 2-OH-myristate, has been described in *A. baumannii* (92, 101), and *K. pneumoniae* (102). The addition of this polar hydroxyl-group presumably complicates the insertion of the hydrophobic domain of colistin into the membrane, yielding resistance. Furthermore, variants in the O-antigen of *Salmonella enterica* have been observe to facilitate resistance to colistin by hindering the colistin from reaching its target (103).

A more extreme mechanism to mediate resistance to colistin is the complete loss of LPS from the cell. The deletion of the structure through which colistin acts, will render the cell non-susceptible altogether. The specific loss of LPS, as a mechanism of colistin resistance, has thus far only been described in specific strains of *A. baumannii*. The loss of LPS has been linked to the loss of expression of penicillin-binding protein (PBP) 1A, which acts in the polymerization of the peptidoglycan layer in the periplasmic space (40, 104). The exact mechanism through which PBP1A prevents loss of LPS however, is unknown. The loss of LPS is compensated by *A. baumannii* through increased synthesis of membrane components like lipoproteins, and phospholipids (79).

Other mechanisms of resistance, that act outside of LPS, have also been observed. A difference in production of the anionic capsular polysaccharides has been observed to cause colistin resistance in *K. pneumoniae* and *N. meningitidis* (105–108). The overproduction of capsular polysaccharides can shield the outer membrane from coming into contact with colistin (105–107). A contrasting observation however, in which the loss of components of the capsule led to an increased colistin resistance has also been reported (108).

Since the integrity of the inner membrane is crucial to survival of Gram-negative bacteria (48), the use of multi-component efflux pumps may increase resilience of the bacterium to the drug (109). The elimination of colistin from the periplasmic space would protect the integrity of the inner membrane, even after colistin succeeds in destabilization of the outer membrane. The effects of efflux pumps on resistance against colistin have been described in *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Stenotropomonas maltophilia* (110–112).

Lastly, the effects of colistin-degrading enzymes have been described. However, the production of these enzymes was not described in Gram-negative bacteria, but in the colistin-producing Gram-positive *P. polymyxa* var. *colistinus* (113), and *Bacillus licheniformis* (114). These hydrolytic enzymes have been found to be inactive against daptomycin and gramicidin, which are other antibiotic compounds with a peptide portion active against Gram-positive bacteria (114). The ability to degrade other types of antimicrobial peptides (including human antimicrobial peptide LL-37), has not been investigated. Thus, it remains an open question why these Gram-positive would produce a degrading enzyme specific for an antibiotic for which they are not susceptible to start with. If these colistin-degrading enzymes however, were to transpose into a mobile genetic element, the impact of such an rearrangement would be a cause of concern, much like the transposition of *mcr* genes into plasmids through the activity of insertion sequence elements (115).

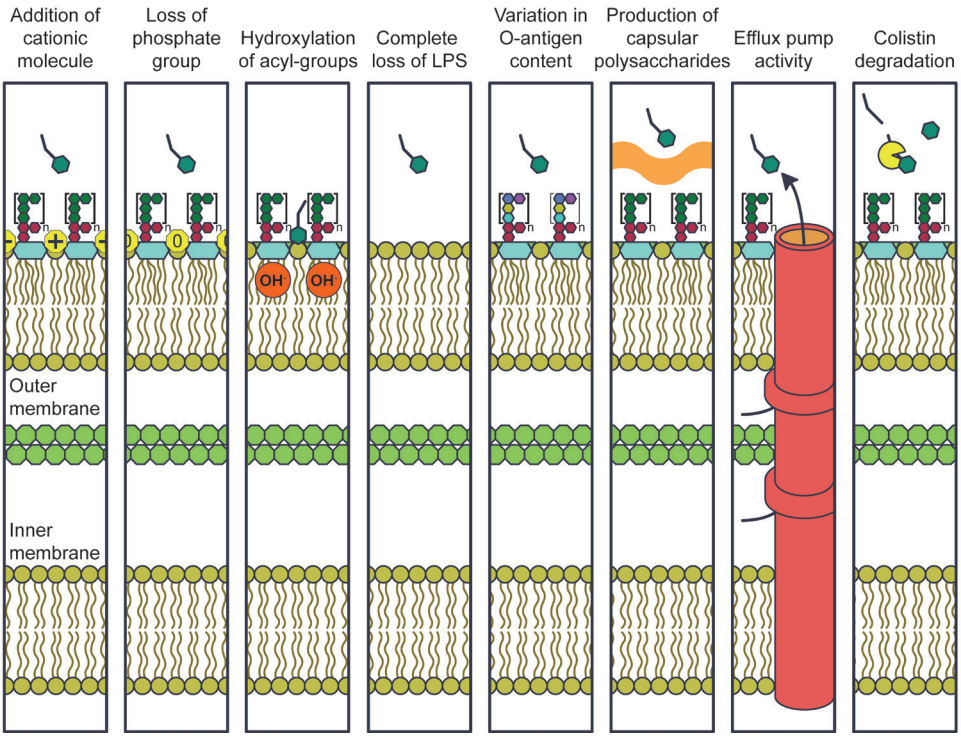

Figure 3: Mechanisms of acquired colistin resistance in Gram-negative bacteria. Mechanisms of acquired colistin resistance that have been described. From left to right: addition of a cationic molecule to lipid A to diminish the net anionic charge; loss of a phosphate group to diminish the net anionic charge; hydroxylation of the acyl groups of the fatty acid groups of lipid A to decrease the insertion of the hydrophobic portion of colistin; complete loss of LPS to delete the structure through which colistin acts; variation in O-antigen to keep colistin from approaching the membrane surface; (over)production of capsular polysaccharides to prevent colistin from bindign to lipid A; expression of multi component efflux pumps to transport colistin from the periplasm to the outside the cell; production of colistin degrading enzymes to inactivate the drug.

The acquisition of one of the mechanisms of resistance listed above, requires changes to the normal physiological processes of Gram-negative bacteria. Two-component regulatory systems are important systems in the development of colistin resistance. Two-component regulatory systems are composed of composed of a sensor kinase, and a transcriptional regulator, and act in response to changes in the environment to increase survival (116). Upon activation of the sensor kinase by binding of specific ligands, the transcriptional regulator will be activated through phosphorylation (116, 117). The activated transcriptional regulator will subsequently influence transcriptional programming by binding to consensus-binding sites, which are generally located in the promoter regions of operons (116). This will mediate the transcriptional reprogramming necessary to enhance survival. Three two-component regulatory systems are considered important in establishing colistin resistance through modification of lipid A and LPS; PmrAB, PhoPQ, and CrrAB (118).

The PmrAB (named BasRS in *E. coli*) two-component regulatory system plays a central role in Gram-negative bacteria, since both the *arn* operon, and *pmrC*, and are transcriptionally regulated by this two-component regulatory system (Figure 4). The normal functioning of the PmrAB two-component regulatory system is aimed at increasing survival when phagocytosed by phagocytes, by detection of compounds associated with phagosomes (e.g. high [Fe^{3+}], and low pH). Modification of lipid A through PmrC, or the Arn-proteins will lead increased survival under influence of the cationic antimicrobial peptides of phagocytes (45, 116). Increased activity of the PhoPQ, and CrrAB, two-component regulatory systems has also been linked to increased PmrAB activity, through the activity of PmrD and CrrC respectively (119). In addition to the presence of their natural ligands, non-synonymous mutations in the genes encoding these three two-component regulatory systems, have been linked to an increased activity (45). Mutation of a negative regulator (e.g. MgrB of PhoPQ), can also lead to increased activity. The increased activity results in the addition of PEtN or L-Ara4N to the lipid A portion, and establishing colistin resistance (45, 119).

Until relatively recently, colistin resistance was thought to be exclusively mediated through chromosomally located mechanisms, and no horizontally transmissible mechanisms of colistin resistance were known. However, the identification of *mcr-1* in 2015 in China, served as a paradigm shift in the mechanisms known to cause colistin resistance (120). A large number of distinct, self-conjugating, plasmids have since been described to carry *mcr* genes. These plasmids have been found in a large range of bacterial species, including ESKAPEE pathogens (115). The *mcr* genes seem to be a particularly important mechanism of colistin resistance in *E. coli*, as it has been found to be the most abundant *mcr*-bearing

species (115, 121, 122). Since the discovery of *mcr-1* in 2015, the number of distinct *mcr* genes identified, has increased to 10, with each different *mcr* gene having a number of variants (123–130). The *mcr* genes all encode for PEtN transferases, and thus transfer PEtN to lipid A, like PmrC described above (131).

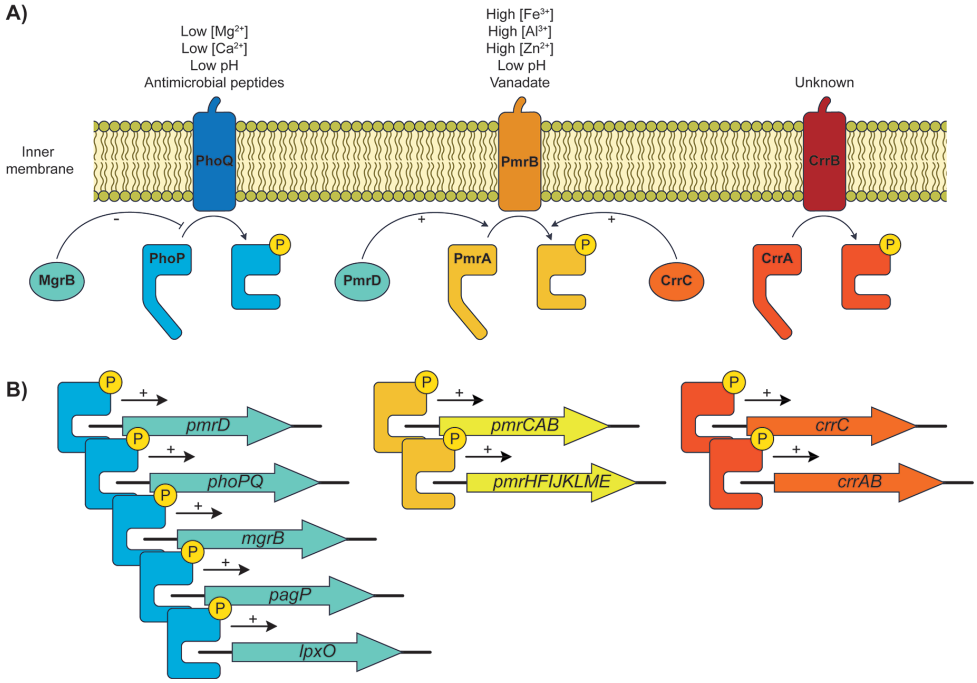

Figure 4: Interaction of two-component regulatory systems involved in lipid A modification, found in Enterobacteriaceae, and their effects on gene expressions. A) Sensor kinases PhoQ, PmrB, and CrrB are located in the inner membrane of Gram-negative bacteria, and are activated by specific ligands present in the periplasm. Upon activation, they activate the transcriptional regulator counterpart in their specific two-component regulatory system through phosphorylation. The activity of PmrA in the PmrAB two-component regulatory system is positively controlled by PmrD and CrrC, independent of PmrB activity. The activity of the PhoPQ two-component regulatory system is negatively controlled by MgrB. The CrrAB two-component regulatory system is only found in select groups of *K. pneumoniae* (132). The effect of PmrD on PmrA not present in *E. coli*, since this effect is effectively negated by the increased dephosphorylation of PmrA by PmrB in *E. coli* (133). The ligands that activate CrrB are not known. **B)** Activation of the transcriptional regulator in the two-component regulatory systems, leads to the upregulation of transcription by binding of the transcriptional regulatory to the promoter region of chromosomally located genes, and subsequent increased activity of RNA polymerases. PhoP activates transcription of, amongst others, *pmrD*, *phoPQ*, *mgrB*, *pagP*, and *lpxO*. PmrA activates transcription of *pmrCAB*, and *pmrHFIJKLME*. CrrA will activate transcription of *crrAB*, and *crrC*. LpxO is not present in *E. coli*. Only operons that apply to this thesis are depicted

Present-day problems in, and solutions for, the development of novel antibiotics

The revival of colistin as a drug of last-resort to treat infections with multidrug-resistant bacteria, stems from the lack of development of novel antibiotics. From the start of the 20th century to the end of the 1980s, the discovery and clinical development of novel classes of antibiotics was sufficient to overcome the development of antibiotic resistance (134–136). However, as most pharmaceutical companies have stopped their antibiotic-development programs, the number of antibiotics being developed for clinical use has steadily declined over the last decades (134, 135, 137). Since the 1980s, only a single class of antibiotics with novel a mechanism of action has been developed; the oxazolidinones (138). This class does not however, affect Gram-negative bacteria. The current pipeline of antibiotics in clinical development otherwise largely contains analogs of existing classes of antibiotics (139, 140). Today, the rate of development of antibiotics with novel mechanisms of action is insufficient to counter the development and spread of multidrug-resistant (particularly Gram-negative) bacteria (139, 140).

The decline in development of antibiotics is mainly caused by the low profitability of antibiotic development. A recent analysis has shown that the net present value of an antibiotic, defined as the total net profit the drug will generate over time, is only $50 million (137), whilst the development costs have been estimated to amount to more than $1.5 billion in 2011 ($1.7 billion in 2019) (141). The decline in the profitability of antibiotics has come through the combination of a steep rise in the expenses needed to get a compound to the market, and the lack of a proportional rise in the revenues of a drug.

The main cause of the steep increase in costs are the increasingly extensive clinical trials needed to deliver the increasing burden of clinic data (e.g. regarding safety) demanded by the regulatory agencies for approval of the drug (142). When considering revenue, there are multiple inherent problems regarding antibiotics which make it hard to generate revenue from them. Antibiotics are generally used for short periods of time, and curative use, contrasting sharply to drugs developed for chronic use (e.g. anti-depressants, or lipid-lowering drugs). Furthermore, the price of antibiotics is also relatively low compared to other types of novel drugs. In addition, an antibiotic will eventually fall into disuse, because of the assured development and spread of resistance against it. Lastly, upon approval of a novel antibiotic, there is a tendency by medical professionals to shelf the antibiotic, to be appropriated for use when other antibiotics fail, limiting its use within the patentable period (137).

These economic reasons have led to many pharmaceutical companies reducing, or completely ending, their efforts to develop novel antimicrobial agents (142). In addition, because the attrition rate of novel products in antibiotic development is high, the number of antibiotics that will be approved in the near future is low (140). Because of the substantial effects that an inability to treat multidrug-resistant bacteria would have on the ability of medical professionals to treat common infections (e.g. urinary tract infections), and to perform routine medical procedures (e.g. hip arthroplasty, or caesarean section), governmental organizations have taken more action in response to the development of multidrug-resistant pathogens. As a response to the dwindling number of antibiotics that may be used against multidrug-resistant pathogens, the World Health Organization (WHO) has defined a priority list of pathogens for which novel antibiotics are specifically needed, due to the threat these pathogens pose to public health (143, 144). In addition, through initiatives like the Global Antibiotic Research and Development Partnership (GARDP) by the WHO (145), and the Innovative Medicines Initiative (IMI) and Joint Programming Initiative on AMR (JPIAMR) by the European Union (146), governmental organizations and industrial partners have created novel initiatives to develop novel, and improve existing, treatments for antibiotic-resistant bacteria through public-private partnerships.

Scope and outline of this thesis

Colistin plays a vital role as a last-resort drug in the treatment of multidrug-resistant Gram-negative bacteria. Considering the lack of development of novel antibiotics active against these types of bacteria, the development of resistance against colistin is a reason for increasing concern. However, there is currently a gap in our understanding of the evolution and mechanisms of colistin resistance in clinically relevant Gram-negative bacteria. In this thesis, we aim to elucidate the mechanisms and evolution of colistin resistance in Enterobacteriaceae. Through the use of clinically relevant strains, we investigate the evolution of, and mechanisms through which colistin resistance may occur. In addition, we study the ability of these bacteria to spread amongst patients, and the impact of colistin resistance on fitness and virulence characteristics.

In **Chapter 2** we investigated the prevalence and mechanisms of colistin resistance in *Escherichia* strains isolated from bloodstream infections, in a tertiary hospital in the Netherlands. We collected ten non-clonally related, colistin-resistant *E. coli* isolates, isolated from bloodstream infections from patient in a tertiary hospital in The Netherlands. We show the low prevalence of colistin resistance, and *mcr* carriage, in the *E. coli* strains isolated from bloodstream infections in this

hospital. We show the modification of lipid A with phosphoethanolamine in the nosocomial stains as a mechanism to diminish electrostatic charges on lipid A. Through a combination of whole genome sequencing, comparative genomics, and construction of transgene insertion mutants we could show that mutations in the genes encoding the BasRS two-component regulatory system contributed to colistin resistance in four of these nosocomial *E. coli* strains.

In **Chapter 3** we investigated the mechanisms of colistin resistance in Enterobacteriaceae isolated from ICU-admitted patients who received the colistin-containing SDD treatment. By collecting colistin-resistant strains through rectal swabs, we showed transient gut colonisation in these patients. Through whole genome sequencing, comparative genomics, and the construction of transgene insertion mutants, we showed the mutations through which Enterobacteriaceae can develop colistin resistance *in vivo*. We observed that there was no clonal relationship between the colistin-resistant strains, outside of those isolated from the same patient. In addition, we show in-patient microevolution of colistin resistance, by analysing a group of colistin-resistant *E. coli* strains isolated from the same patient. We observe that a clonal population of colistin-resistant *E. coli* strains may develop resistance through multiple evolutionary pathways.

In **Chapter 4** we used a collection of isogenic colistin-susceptible, and colistin-resistant ESBL-producing *K. pneumoniae* strains isolated from ICU-admitted patients, receiving SDD treatment. We show the *de novo* development of colistin resistance in these strains, through a diverse set of chromosomal mutations (leading to substitutions in PhoQ, LpxM, and YciM, and inactivation of *mgrB*). We confirm the role of these mutations in colistin resistance through *in trans* complementation experiments and susceptibility determinations.

In **Chapter 5** we performed an *in vitro* evolution experiment by passaging, cultures of nosocomial *K. pneumoniae* complex strains (three *K. pneumoniae sensu stricto*, and one *K. variicola* subsp. *variicola*) in increasing concentrations of colistin. We demonstrate the multiple evolutionary pathways through which nosocomial *K. pneumoniae* complex strains can develop resistance to colistin. Through population sequencing we observe the swift, and selective fixation of mutations located in genes associated with LPS modification and assembly, and capsule synthesis within the evolving populations. We show the absence of a general impact on fitness in these clinically relevant *K. pneumoniae* complex strains upon development of colistin resistance.

Chapter 6 discusses the implications of the found evolutionary mechanisms through which Enterobacteriaceae may develop resistance against colistin, in the context of current literature.

References

1. Kaper JB, Nataro JP, Mobley HLT. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140.
2. Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, Jenney A, Connor TR, Hsu LY, Severin J, Brisse S, Cao H, Wilksch J, Gorrie C, Schultz MB, Edwards DJ, Nguyen K V, Nguyen TV, Dao TT, Mensink M, Minh VL, Nhu NTK, Schultsz C, Kuntaman K, Newton PN, Moore CE, Strugnell RA, Thomson NR. 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc Natl Acad Sci U S A 112:E3574–E3581.
3. Rodrigues C, Passet V, Rakotondrasoa A, Diallo TA, Criscuolo A, Brisse S. 2019. Description of *Klebsiella africanensis* sp. nov., *Klebsiella variicola* subsp. *tropicalensis* subsp. nov. and *Klebsiella variicola* subsp. *variicola* subsp. nov. Res Microbiol 170:165–170.
4. Tandogdu Z, Wagenlehner FME. 2016. Global epidemiology of urinary tract infections. Curr Opin Infect Dis 29:73–79.
5. World Health Organization. 2014. Antimicrobial resistance. Global report on surveillance.
6. Diekema DJ, Hsueh P-R, Mendes RE, Pfaller MA, Rolston K V., Sader HS, Jones RN. 2019. The microbiology of bloodstream infection: 20-year trends from the SENTRY antimicrobial surveillance program. Antimicrob Agents Chemother 63:e00355-19.
7. de Kraker MEA, Jarlier V, Monen JCM, Heuer OE, van de Sande N, Grundmann H. 2013. The changing epidemiology of bacteraemias in Europe: trends from the European Antimicrobial Resistance Surveillance System. Clin Microbiol Infect 19:860–868.
8. Follador R, Heinz E, Wyres KL, Ellington MJ, Kowarik M, Holt KE, Thomson NR. 2016. The diversity of *Klebsiella pneumoniae* surface polysaccharides. Microb Genomics 2:e000073.
9. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48:1–12.
10. Llaca-Díaz JM, Mendoza-Olazarán S, Camacho-Ortiz A, Flores S, Garza-González E. 2013. One-year surveillance of ESKAPE pathogens in an intensive care unit of Monterrey, Mexico. Chemotherapy 58:475–481.
11. Iredell J, Brown J, Tagg K. 2016. Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications. Br Med J 351:h6420.
12. Castanheira M, Deshpande LM, Mendes RE, Canton R, Sader HS, Jones RN. 2019. Variations in the occurrence of resistance phenotypes and carbapenemase genes among enterobacteriaceae isolates in 20 years of the SENTRY antimicrobial surveillance program. Open Forum Infect Dis 6:S23–S33.
13. European Centre for Disease Prevention and Control. 2019. Surveillance of antimicrobial resistance in Europe 2018, Surveillance of antimicrobial resistance in Europe.
14. Navon-Venezia S, Kondratyeva K, Carattoli A. 2017. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol Rev 41:252–275.
15. Fevre C, Passet V, Weill FX, Grimont PAD, Brisse S. 2005. Variants of the *Klebsiella pneumoniae* OKP chromosomal beta-lactamase are divided into two main groups, OKP-A and OKP-B. Antimicrob Agents Chemother 49:5149–5152.
16. Lee C-R, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. 2016. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: epidemiology, genetic context, treatment options, and detection methods. Front Microbiol 7:895.
17. Grundmann H, Glasner C, Albiger B, Aanensen DM, Tomlinson CT, Andrasević AT, Cantón R, Carmeli Y, Friedrich AW, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Nordmann P, Poirel L, Rossolini GM, Seifert H, Vatopoulos A, Walsh T, Woodford N, Monnet DL, Apfalter P, Hartl R, Glupczynski Y, Huang TD, Strateva T, Marteva-Proevska Y, Andraševic AT, Butic I, Pieridou-Bagatzouni D, Maikanti-Charalampous P, Hrabak J, Zemlickova H, Hammerum A, Jakobsen L, Ivanova M, Pavelkovich A, Jalava J, Österblad M, Vaux S, Dortet L, Kaase M, Gatermann SG, Vatopoulos A, Tryfinopoulou K, Tóth Á, Jánvári L, Boo TW, McGrath E, Pantosti A, Monaco M, Balode A, Saule M, Miciuleviciene J, Mierauskaite A, Perrin-Weniger M, Reichert P, Nestorova N, Debattista S, Zabicka D, Literacka E, Caniça M, Manageiro V, Damian M, Lixandru B, Nikš M, Schreterova E, Pirš M, Cerar T, Oteo J, Aracil B, Giske CG, Sjöström K, Woodford N, Hopkins K, Wiuff C, Brown DJ, Hardarson H, Samuelsen Ø, Haldorsen B, Koraqi A, Lacej D, Raka L, Kurti A, Mijovic G, Lopicic M, Jelesic Z, Trudic A, Kraftandzieva A, Trajkovska-Dokic E, Gür D, Cakar A, Carmeli Y, Adler A. 2017. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE): a prospective, multinational study. Lancet Infect Dis 17:153–163.

18. Castanheira M, Mendes RE, Woosley LN, Jones RN. 2011. Trends in carbapenemase-producing *Escherichia coli* and *Klebsiella* spp. from Europe and the Americas: Report from the SENTRY antimicrobial surveillance programme (2007-09). J Antimicrob Chemother 66:1409–1411.
19. McNally A, Kallonen T, Connor C, Abudahab K, Aanensen DM, Horner C, Peacock SJ, Parkhill J, Croucher NJ, Corander J. 2019. Diversification of colonization factors in a multidrug-resistant *Escherichia coli* lineage evolving under negative frequency-dependent selection. mBio 10:e00644-19.
20. Kallonen T, Brodrick HJ, Harris SR, Corander J, Brown NM, Martin V, Peacock SJ, Parkhill J. 2017. Systematic longitudinal survey of invasive *Escherichia coli* in England demonstrates a stable population structure only transiently disturbed by the emergence of ST131. Genome Res 27:1437–1449.
21. Moradigaravand D, Martin V, Peacock SJ, Parkhill J. 2017. Evolution and epidemiology of multidrug-resistant *Klebsiella pneumoniae* in the United Kingdom and Ireland. mBio 8:e01976-16.
22. Potron A, Kalpoe J, Poirel L, Nordmann P. 2011. European dissemination of a single OXA-48-producing *Klebsiella pneumoniae* clone. Clin Microbiol Infect 17:E24–E26.
23. Galani I, Karaiskos I, Karantani I, Papoutsaki V, Maraki S, Papaioannou V, Kazila P, Tsorlini H, Charalampaki N, Toutouza M, Vagiakou H, Pappas K, Kyratsa A, Kontopoulou K, Legga O, Petinaki E, Papadogeorgaki H, Chinou E, Souli M, Giamarellou H. 2018. Epidemiology and resistance phenotypes of carbapenemase-producing *Klebsiella pneumoniae* in Greece, 2014 to 2016. Eurosurveillance 23.
24. Yang Y, Zhang A, Lei C, Wang H, Guan Z, Xu C, Liu B, Zhang D, Li Q, Jiang W, Pan Y, Yang C. 2015. Characteristics of plasmids coharboring 16S rRNA methylases, CTX-M, and virulence factors in *Escherichia coli* and *Klebsiella pneumoniae* isolates from chickens in China. Foodborne Pathog Dis 12:873–880.
25. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, Goossens H, Laxminarayan R. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci 115:E3463–E3470.
26. Giakoupi P, Maltezou H, Polemis M, Pappa O, Saroglou G, Vatopoulos A. 2008. KPC-2 producing *Klebsiella pneumoniae* infections in Greek hospitals are mainly due to a hyperepidemic clone. Eurosurveillance 14.
27. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Miriagou V, Naas T, Rossolini GM, Samuelsen Ø, Seifert H, Woodford N, Nordmann P. 2012. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin Microbiol Infect 18:413–431.
28. Kontopoulou K, Protonotariou E, Vasilakos K, Kriti M, Koteli A, Antoniadou E, Sofianou D. 2010. Hospital outbreak caused by *Klebsiella pneumoniae* producing KPC-2 β-lactamase resistant to colistin. J Hosp Infect 76:70–73.
29. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Eurosurveillance 19:20939.
30. Parisi SG, Bartolini A, Santacatterina E, Castellani E, Ghirardo R, Berto A, Franchin E, Menegotto N, De Canale E, Tommasini T, Rinaldi R, Basso M, Stefani S, Palù G. 2015. Prevalence of *Klebsiella pneumoniae* strains producing carbapenemases and increase of resistance to colistin in an Italian teaching hospital from January 2012 to December 2014. BMC Infect Dis 15:244.
31. Marchaim D, Chopra T, Pogue JM, Perez F, Hujer AM, Rudin S, Endimiani A, Navon-Venezia S, Hothi J, Slim J, Blunden C, Shango M, Lephart PR, Salimnia H, Reid D, Moshos J, Hafeez W, Bheemreddy S, Chen TY, Dhar S, Bonomo RA, Kaye KS. 2011. Outbreak of colistin-resistant, carbapenem-resistant *Klebsiella pneumoniae* in Metropolitan Detroit, Michigan. Antimicrob Agents Chemother 55:593–599.
32. Li J, Nation RL, Kaye KS. 2019. Polymyxin antibiotics: from laboratory bench to bedside.
33. Cai Y, Lee W, Kwa AL. 2015. Polymyxin B versus colistin: an update. Expert Rev Anti Infect Ther 7210:1–17.
34. Velkov T, Thompson PE, Nation RL, Li J. 2010. Structure-activity relationships of polymyxin antibiotics. J Med Chem 53:1898–1916.
35. Suzuki T, Hayashi K, Fujikawa K, Tsukamoto K. 1965. The chemical structure of polymyxin E: the identities of polymyxin E1 with colistin A and of polymyxin E2 with colistin B*. J Biochem 57:226–227.
36. Li J, Nation RL, Milne RW, Turnidge JD, Coulthard K. 2005. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. Int J Antimicrob Agents 25:11–25.
37. Roberts KD, Azad MAK, Wang J, Horne AS, Thompson PE, Nation RL, Velkov T, Li J. 2016. Antimicrobial activity and toxicity of the major lipopeptide components of polymyxin B and colistin: last-line antibiotics against multidrug-resistant Gram-negative bacteria. ACS Infect Dis 1:568–575.
38. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. Clin Infect Dis 40:1333–1341.

39. Sabnis A, Klöckner A, Becce M, Evans LE, Furniss RCD, Mavridou DAI, Stevens MM, Edwards AM. 2018. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. bioRxiv 479618v2.
40. Moffatt JH, Harper M, Harrison P, Hale JDF, Vinogradov E, Seemann T, Henry R, Crane B, Michael FS, Cox AD, Adler B, Nation RL, Li J, Boyce JD. 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother 54:4971–4977.
41. Jayol A, Saly M, Nordmann P, Menard A, Poirel L, Dubois V. 2017. *Hafnia*, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. J Antimicrob Chemother 72:2507–2511.
42. Velasco J, Romero C, Leiva J, Diazf R. 1998. Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. Int J Syst Bacteriol 48:759–768.
43. Göker T, Aşık RZ, Yılmaz MB, Çelik İ, Tekiner A. 2017. *Sphingomonas paucimobilis*: A rare infectious agent found in cerebrospinal fluid. J Korean Neurosurg Soc 60:481–483.
44. Vaz-Moreira I, Nunes OC, Manaia CM. 2011. Diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolates from drinking water. Appl Environ Microbiol 77:5697–5706.
45. Olaitan AO, Morand S, Rolain J-M. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643.
46. Hancock REW, Chapple DS. 1999. Peptide antibiotics. Antimicrob Agents Chemother 43:1317–1323.
47. Schindler M, Osborn MJ. 1979. Interaction of divalent cations and polymyxin B with lipopolysaccharide. Biochemistry 18:4425–4430.
48. Heesterbeek DA, Bardoel BW, Parsons ES, Bennett I, Ruyken M, Doorduijn DJ, Gorham RD, Berends ET, Pyne AL, Hoogenboom BW, Rooijakkers SH. 2019. Bacterial killing by complement requires membrane attack complex formation via surface-bound C5 convertases. EMBO J 38.
49. Giuliani A, Pirri G, Nicoletto SF. 2007. Antimicrobial peptides: an overview of a promising class of therapeutics. Cent Eur J Biol 2:1–33.
50. Brogden KA. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–50.
51. Putker F, Bos MP, Tommassen J. 2015. Transport of lipopolysaccharide to the Gram-negative bacterial cell surface. FEMS Microbiol Rev 39:985–1002.
52. de Smet AMGA, Kluytmans JAJW, Cooper BS, Mascini EM, Benus RFJ, van der Werf TS, van der Hoeven JG, Pickkers P, Bogaers-Hofman D, van der Meer NJM, Bernards AT, Kuijper EJ, Joore JCA, Leverstein-van Hall MA, Bindels AJGH, Jansz AR, Wesselink RMJ, de Jongh BM, Dennesen PJW, van Asselt GJ, te Velde LF, Frenay IHME, Kaasjager K, Bosch FH, van Iterson M, Thijsen SFT, Kluge GH, Pauw W, de Vries JW, Kaan JA, Arends JP, Aarts LPHJ, Sturm PDJ, Harinck HIJ, Voss A, Uijtendaal E V, Blok HEM, Thieme Groen ES, Pouw ME, Kalkman CJ, Bonten MJM. 2009. Decontamination of the digestive tract and oropharynx in ICU patients. N Engl J Med 360:20–31.
53. Oostdijk EAN, Kesecioglu J, Schultz MJ, Visser CE, De Jonge E, Van Essen EHR, Bernards AT, Purmer I, Brimicombe R, Bergmans D, Van Tiel F, Bosch FH, Mascini E, Van Griethuysen A, Bindels A, Jansz A, Van Steveninck FAL, Van Der Zwet WC, Fijen JW, Thijsen S, De Jong R, Oudbier J, Raben A, Van Der Vorm E, Koeman M, Rothbarth P, Rijkeboer A, Gruteke P, Hart-Sweet H, Peerbooms P, Winsser LJ, Van Elsacker-Niele AMW, Demmendaal K, Brandenburg A, De Smet AMGA, Bonten MJM. 2014. Effects of decontamination of the oropharynx and intestinal tract on antibiotic resistance in ICUs a randomized clinical trial. J Am Med Assoc 312:1429–1437.
54. Vincent J-L, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, Moreno R, Lipman J, Gomersall C, Sakr Y, Reinhart K, for the EPIC II Group of Investigators. 2009. International study of the prevalence and outcomes of infection in intensive care units. J Am Med Assoc 302:2323–2329.
55. Fridkin SK, Welbel SF, Weinstein RA. 1997. Magnitude and prevention of nosocomial infections in the intensive care unit. Infect Dis Clin North Am 11:479–496.
56. van der Waaij D, Manson WL, Arends JP, de Vries-Hospers HG. 1990. Clinical use of selective decontamination: the concept. Intensive Care Med 16:212–216.
57. Wittekamp BH, Plantinga NL, Cooper BS, Lopez-Contreras J, Coll P, Mancebo J, Wise MP, Morgan MPG, Depuydt P, Boelens J, Dugernier T, Verbelen V, Jorens PG, Verbrugghe W, Malhotra-Kumar S, Damas P, Meex C, Leleu K, Van Den Abeele AM, Gomes Pimenta De Matos AF, Fernández Méndez S, Vergara Gomez A, Tomic V, Sifrer F, Villarreal Tello E, Ruiz Ramos J, Aragao I, Santos C, Sperning RHM, Coppadoro P, Nardi G, Brun-Buisson C, Bonten MJM. 2018. Decontamination strategies and bloodstream infections with antibiotic-resistant microorganisms in ventilated patients: a randomized clinical trial. J Am Med Assoc 320:2087–2098.

58. Oostdijk EAN, Smits L, De Smet AMGA, Leverstein-Van Hall MA, Kesecioglu J, Bonten MJM. 2013. Colistin resistance in Gram-negative bacteria during prophylactic topical colistin use in intensive care units. Intensive Care Med 39:653–660.
59. Halaby T, al Naiemi N, Kluytmans J, van der Palen J, Vandenbroucke-Grauls CMJE. 2013. Emergence of colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. Antimicrob Agents Chemother 57:3224–3229.
60. Bastin AJ, Ryanna KB. 2009. Use of selective decontamination of the digestive tract in United Kingdom intensive care units. Anaesthesia 64:46–49.
61. Kempf I, Jouy E, Chauvin C. 2016. Colistin use and colistin resistance in bacteria from animals. Int J Antimicrob Agents 48:598–606.
62. Tuo H, Yang Y, Tao X, Liu D, Li Y, Xie X, Li P, Gu J, Kong L, Xiang R, Lei C, Wang H, Zhang A. 2018. The prevalence of colistin resistant strains and antibiotic resistance gene profiles in Funan river, China. Front Microbiol 9:3094.
63. Shen C, Zhong L, Yang Y, Doi Y, Paterson DL, Stoesser N, Ma F, El-Sayed Ahmed MAE, Feng S, Huang S, Li H, Huang X, Wen X, Zhao Z, Lin M, Chen G, Liang W, Liang Y, Xia Y, Dai M, Chen D, Zhang L, Liao K, Tian G. 2020. Dynamics of *mcr-1* prevalence and *mcr-1*-positive *Escherichia coli* after the cessation of colistin use as a feed additive for animals in China: a prospective cross-sectional and whole genome sequencing based molecular epidemiological study. Lancet Microbe 1:e34-e43.
64. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. 2012. Colistin: an update on the antibiotic of the 21st century. Expert Rev Anti Infect Ther 10:917–934.
65. Ordooei Javan A, Shokouhi S, Sahraei Z. 2015. A review on colistin nephrotoxicity. Eur J Clin Pharmacol 71:801–810.
66. Hartzell JD, Neff R, Ake J, Howard R, Olson S, Paolino K, Vishnepolsky M, Weintrob A, Wortmann G. 2009. Nephrotoxicity associated with intravenous colistin (colistimethate sodium) treatment at a tertiary care medical center. Clin Infect Dis 48:1724–1728.
67. Gai Z, Samodelov SL, Kullak-Ublick GA, Visentin M. 2019. Molecular mechanisms of colistin-enduced nephrotoxicity. Molecules 24:653.
68. Heybeli C, Oktan MA, Çavdar Z. 2019. Rat models of colistin nephrotoxicity: previous experimental researches and future perspectives. Eur J Clin Microbiol Infect Dis 38:1387–1393.
69. Ahmed MU, Velkov T, Zhou QT, Fulcher AJ, Callaghan J, Zhou F, Chan K, Azad MAK, Li J. 2018. Intracellular localization of polymyxins in human alveolar epithelial cells. J Antimicrob Chemother 74:48–57.
70. Lewis JR, Lewis SA. 2004. Colistin interactions with the mammalian urothelium. Am J Physiol - Cell Physiol 286:913–922.
71. Berg JR, Spilker CM, Lewis SA. 1996. Effects of polymyxin B on mammalian urinary bladder. J Membr Biol 154:119–130.
72. Dai C, Li J, Tang S, Li J, Xiao X. 2014. Colistin-induced nephrotoxicity in mice involves the mitochondrial, death receptor, and endoplasmic reticulum pathways. Antimicrob Agents Chemother 58:4075–4085.
73. Azzopardi EA, Ferguson EL, Thomas DW. 2013. Colistin past and future: a bibliographic analysis. J Crit Care 28:219.e13–9.
74. Falagas ME, Kasiakou SK. 2006. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit care 10:R27.
75. Hori Y, Aoki N, Kuwahara S, Hosojima M, Kaseda R, Goto S, Iida T, De S, Kabasawa H, Kaneko R, Aoki H, Tanabe Y, Kagamu H, Narita I, Kikuchi T, Saito A. 2017. Megalin blockade with cilastatin suppresses drug-induced nephrotoxicity. J Am Soc Nephrol 28:1783–1791.
76. Ma Z, Wang J, Nation RL, Li J, Turnidge JD, Coulthard K, Milne RW. 2009. Renal disposition of colistin in the isolated perfused rat kidney. Antimicrob Agents Chemother 53:2857–2864.
77. Korucu B, Unal I, Pekcan M, Inkaya AC, Yeter H, Cetinkaya MA, Kaymaz FF, Unal S, Akova M, Erdem Y. 2019. Ultrastructural evaluation of urine alkalinization versus hydration on colistin-induced nephrotoxicity. Hum Exp Toxicol 38:1366–1377.
78. Zhang G, Meredith TC, Kahne D. 2013. On the essentiality of lipopolysaccharide to Gram-negative bacteria. Curr Opin Microbiol 16:779–785.
79. Henry R, Vithanage N, Harrison P, Seemann T, Coutts S, Moffatt JH, Nation RL, Li J, Harper M, Adler B, Boyce JD. 2012. Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-β-1,6-N-acetylglucos. Antimicrob Agents Chemother 56:59–69.
80. Bertani B, Ruiz N. 2017. Function and biogenesis of lipoposaccharides. EcoSal Plus 176:139–148.
81. Whitfield C, Trent MS. 2014. Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem

83:99–128.
82. Mahalakshmi S, Sunayana MR, Saisree L, Reddy M. 2014. *yciM* is an essential gene required for regulation of lipopolysaccharide synthesis in *Escherichia coli*. Mol Microbiol 91:145–157.
83. Henderson JC, Zimmerman SM, Crofts AA, Boll JM, Kuhns LG, Herrera CM, Trent MS. 2016. The power of asymmetry: architecture and assembly of the Gram-negative outer membrane lipid bilayer. Annu Rev Microbiol 70:255–278.
84. Moffatt JH, Harper M, Mansell A, Crane B, Fitzsimons TC, Nation RL, Li J, Adler B, Boyce JD. 2013. Lipopolysaccharide-deficient *Acinetobacter baumannii* shows altered signaling through host Toll-like receptors and increased susceptibility to the host antimicrobial peptide LL-37. Infect Immun 81:684–689.
85. Sprong T, Stikkelbroeck N, van der Ley P, Steeghs L, van Alphen L, Klein N, Netea MG, van der Meer JW, van Deuren M. 2001. Contributions of *Neisseria meningitidis* LPS and non-LPS to proinflammatory cytokine response. J Leukoc Biol 70:283–288.
86. Peng D, Hong W, Choudhury BP, Carlson RW, Gu XX. 2005. *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. Infect Immun 73:7569–7577.
87. Kawasaki S, Moriguchi R, Sekiya K, Nakai T, Ono E, Kume K, Kawahara K. 1994. The cell envelope structure of the lipopolysaccharide-lacking Gram-negative bacterium *Sphingomonas paucimobilis*. J Bacteriol 176:284–290.
88. Pelletier MR, Casella LG, Jones JW, Adams MD, Zurawski D V., Hazlett KRO, Doi Y, Ernst RK. 2013. Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. Antimicrob Agents Chemother 57:4831–4840.
89. Xu Y, Wei W, Lei S, Lin J, Srinivas S, Feng Y. 2018. An evolutionarily conserved mechanism for intrinsic and transferable polymyxin resistance. mBio 9:e02317-17.
90. Wang X, Ribeiro AA, Guan Z, Raetz CRH. 2009. Identification of undecaprenyl phosphate-β-D-Galactosamine in *Francisella novicida* and its function in lipid A modification. Biochemistry 48:1162–1172.
91. Choi MJ, Ko KS. 2015. Loss of hypermucoviscosity and increased fitness cost in colistin-resistant *Klebsiella pneumoniae* sequence type 23 strains. Antimicrob Agents Chemother 59:6763–6773.
92. Mills G, Dumigan A, Kidd T, Hobley L, Bengoechea JA. 2017. Identification and characterization of two *Klebsiella pneumoniae lpxL* lipid A late acylstransferases and their role in virulence. Infect Immun 85:e00068-17.
93. Leung LM, Cooper VS, Rasko DA, Guo Q, Pacey MP, McElheny CL, Mettus RT, Yoon SH, Goodlett DR, Ernst RK, Doi Y. 2017. Structural modification of LPS in colistin-resistant, KPC-producing *Klebsiella pneumoniae*. J Antimicrob Chemother 72:3035–3042.
94. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Høiby N, Moskowitz SM. 2011. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 55:5761–5769.
95. Dortet L, Potron A, Bonnin RA, Plesiat P, Naas T, Filloux A, Larrouy-Maumus G. 2018. Rapid detection of colistin resistance in *Acinetobacter baumannii* using MALDI-TOF-based lipidomics on intact bacteria. Sci Rep 8:16910.
96. Janssen AB, Bartholomew TL, Marciszewska NP, Bonten MJM, Willems RJL, Bengoechea JA, van Schaik W. 2020. Nonclonal emergence of colistin resistance associated with mutations in the BasRS two-component system in Escherichia coli bloodstream isolates. mSphere 5:e00143-20
97. Liu Y, Chandler CE, Leung LM, Mcelheny CL, Mettus RT, Shanks RMQ, Liu J, Goodlett DR, Ernst RK. 2017. Structural modification of lipopolysaccharide conferred by *mcr-1* in Gram-negative ESKAPE pathogens. Antimicrob Agents Chemother 61:e00580-17.
98. Kong Q, Six DA, Liu Q, Gu L, Wang S, Alamuri P, Raetz CRH, Curtiss R. 2012. Phosphate groups of lipid A are essential for *Salmonella enterica* serovar Typhimurium virulence and affect innate and adaptive immunity. Infect Immun 80:3215–3224.
99. Karbarz MJ, Kalb SR, Cotter RJ, Raetz CRH. 2003. Expression cloning and biochemical characterization of a *Rhizobium leguminosarum* lipid A 1-phosphatase. J Biol Chem 278:39269–39279.
100. Wang X, McGrath SC, Cotter RJ, Raetz CRH. 2006. Expression cloning and periplasmic orientation of the *Francisella novicida* lipid A 4′-phosphatase LpxF. J Biol Chem 281:9321–9330.
101. Bartholomew TL, Kidd TJ, Sá Pessoa J, Conde Álvarez R, Bengoechea JA. 2019. 2-hydroxylation of *Acinetobacter baumannii* lipid A contributes to virulence. Infect Immun 87:e00066-19.
102. Llobet E, Martínez-Moliner V, Moranta D, Dahlström KM, Regueiro V, Tomás A, Cano V, Pérez-Gutiérrez C, Frank CG, Fernández-Carrasco H, Insua JL, Salminen TA, Garmendia J, Bengoechea JA. 2015. Deciphering

tissue-induced *Klebsiella pneumoniae* lipid A structure. Proc Natl Acad Sci U S A 112:E6369–E6378.
103. Ricci V, Zhang D, Teale C, Piddock LJV. 2020. The O-antigen epitope governs susceptibility to colistin in *Salmonella enterica*. mBio 11:e02831-19.
104. Boll JM, Crofts AA, Peters K, Cattoir V, Vollmer W, Davies BW, Trent MS. 2016. A penicillin-binding protein inhibits selection of colistin-resistant, lipooligosaccharide-deficient *Acinetobacter baumannii*. Proc Natl Acad Sci 113:E6228–E6237.
105. Llobet E, Tomás JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154:3877–3886.
106. Campos MA, Vargas MA, Regueiro V, Llompart CM, Albertí S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun 72:7107–7114.
107. Spinosa MR, Progida C, Talà A, Cogli L, Alifano P, Bucci C. 2007. The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. Infect Immun 75:3594–3603.
108. Pal S, Verma J, Mallick S, Rastogi SK, Kumar A, Ghosh AS. 2019. Absence of the glycosyltransferase WcaJ in *Klebsiella pneumoniae* ATCC13883 affects biofilm formation, increases polymyxin resistance and reduces murine macrophage activation. Microbiology 165:891–904.
109. Li XZ, Plésiat P, Nikaido H. 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 28:337–418.
110. Cheng Y-H, Lin T-L, Lin Y-T, Wang J-T. 2018. A putative RND-type efflux pump, H239_3064, contributes to colistin resistance through CrrB in *Klebsiella pneumoniae*. J Antimicrob Chemother 73:1509–1516.
111. Srinivasan VB, Rajamohan G. 2013. KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. Antimicrob Agents Chemother 57:4449–4462.
112. Ni W, Li Y, Guan J, Zhao J, Cui J, Wang R, Liu Y. 2016. Effects of efflux pump inhibitors on colistin resistance in multidrug-resistant Gram-negative bacteria. Antimicrob Agents Chemother 60:3215–3218.
113. Ito-Kagawa M, Koyama Y. 1980. Selective cleavage of a peptide antibiotic, colistin by colistinase. J Antibiot (Tokyo) 33:1551–1555.
114. Yin J, Wang G, Cheng D, Fu J, Qiu J, Yu Z. 2019. Inactivation of polymyxin by hydrolytic mechanism. Antimicrob Agents Chemother 63:e02378-18.
115. Wang R, van Dorp L, Shaw LP, Bradley P, Wang Q, Wang X, Jin L, Zhang Q, Liu Y, Rieux A, Dorai-Schneiders T, Weinert LA, Iqbal Z, Didelot X, Wang H, Balloux F. 2018. The global distribution and spread of the mobilized colistin resistance gene *mcr-1*. Nat Commun 9:1179.
116. Gunn JS. 2008. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol 16:284–290.
117. Richards SM, Strandberg KL, Conroy M, Gunn JS. 2012. Cationic antimicrobial peptides serve as activation signals for the *Salmonella* Typhimurium PhoPQ and PmrAB regulons *in vitro* and *in vivo*. Front Cell Infect Microbiol 2:102.
118. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev 30:557–596.
119. Cheng Y-H, Lin T-L, Lin Y-T, Wang J-T. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 60:3709–3716.
120. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168.
121. Chen K, Chan EWC, Xie M, Ye L, Dong N, Chen S. 2017. Widespread distribution of *mcr-1*-bearing bacteria in the ecosystem, 2015 to 2016. Eurosurveillance 22:17–00206.
122. Shen Z, Wang Y, Shen Y, Shen J, Wu C. 2016. Early emergence of *mcr-1* in *Escherichia coli* from food-producing animals. Lancet Infect Dis 16:293.
123. Xavier B, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. Eurosurveillance 21:30280.
124. Wenjuan Y, Hui L, Yingbo S, Liu Z, Wang S, Shen Z, Zhang R, Walsh TR, Jianzhong S, Wang Y. 2017. Novel plasmid-ediated colistin resistance gene *mcr-3* in *Escherichia coli*. mBio 8:e00543-17.
125. Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G, Magistrali CF. 2017. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and

Belgium, 2015 to 2016. Eurosurveillance 22:30589.
126. Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. J Antimicrob Chemother 72:3317–3324.
127. AbuOun M, Stubberfield EJ, Duggett NA, Kirchner M, Dormer L, Nunez-Garcia J, Randall LP, Lemma F, Crook DW, Teale C, Smith RP, Anjum MF. 2017. *mcr-1* and *mcr-2* variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. J Antimicrob Chemother 72:2745–2749.
128. Yang YQ, Li YX, Lei CW, Zhang AY, Wang HN. 2018. Novel plasmid-mediated colistin resistance gene *mcr-7.1* in *Klebsiella pneumoniae*. J Antimicrob Chemother 73:1791–1795.
129. Wang X, Wang Y, Zhou Y, Li J, Yin W, Wang S, Zhang S, Shen J, Shen Z, Wang Y. 2018. Emergence of a novel mobile colistin resistance gene, *mcr-8*, in NDM-producing *Klebsiella pneumoniae*. Emerg Microbes Infect 7:122.
130. Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, Wiedmanna M. 2019. Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate. mBio 10:e00853-19.
131. Anandan A, Evans GL, Condic-Jurkic K, O'Mara ML, John CM, Phillips NJ, Jarvis GA, Wills SS, Stubbs KA, Moraes I, Kahler CM, Vrielink A. 2017. Structure of a lipid A phosphoethanolamine transferase suggests how conformational changes govern substrate binding. Proc Natl Acad Sci 114:2218–2223.
132. Cain AK, Boinett CJ, Barquist L, Dordel J, Fookes M, Mayho M, Ellington MJ, Goulding D, Pickard D, Wick RR, Holt KE, Parkhill J, Thomson NR. 2018. Morphological, genomic and transcriptomic responses of *Klebsiella pneumoniae* to the last-line antibiotic colistin. Sci Rep 8:9868.
133. Raetz CR, Reynolds MC, Trent SM, Bishop RE. 2007. Lipid A modification in Gram-negative bacteria. Annu Rev Biochem 76:295–329.
134. Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433.
135. Shore CK, Coukell A. 2016. Roadmap for antibiotic discovery. Nat Microbiol 1:16083.
136. Silver LL. 2011. Challenges of antibacterial discovery. Clin Microbiol Rev 24:71–109.
137. Mobarki N, Almerabi B, Hattan A. 2019. Antibiotic Resistance Crisis. Int J Med Dev Ctries 40:561–564.
138. Devasahayam G, Scheld WM, Hoffman PS. 2010. New antibacterial drugs for a new century. Expert Opin Investig Drugs 19:215–234.
139. World Health Organization. 2019. 2019 Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline.
140. World Health Organization. 2019. Antibacterial agents in preclinical development: an open access database.
141. Towse A, Hoyle CK, Goodall J, Hirsch M, Mestre-Ferrandiz J, Rex JH. 2017. Time for a change in how new antibiotics are reimbursed: development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. Health Policy 121:1025–1030.
142. Norrby SR, Nord CE, Finch R. 2005. Lack of development of new antimicrobial drugs: A potential serious threat to public health. Lancet Infect Dis 5:115–119.
143. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N, Aboderin AO, Al-Abri SS, Awang Jalil N, Benzonana N, Bhattacharya S, Brink AJ, Burkert FR, Cars O, Cornaglia G, Dyar OJ, Friedrich AW, Gales AC, Gandra S, Giske CG, Goff DA, Goossens H, Gottlieb T, Guzman Blanco M, Hryniewicz W, Kattula D, Jinks T, Kanj SS, Kerr L, Kieny MP, Kim YS, Kozlov RS, Labarca J, Laxminarayan R, Leder K, Leibovici L, Levy-Hara G, Littman J, Malhotra-Kumar S, Manchanda V, Moja L, Ndoye B, Pan A, Paterson DL, Paul M, Qiu H, Ramon-Pardo P, Rodríguez-Baño J, Sanguinetti M, Sengupta S, Sharland M, Si-Mehand M, Silver LL, Song W, Steinbakk M, Thomsen J, Thwaites GE, van der Meer JW, Van Kinh N, Vega S, Villegas MV, Wechsler-Fördös A, Wertheim HFL, Wesangula E, Woodford N, Yilmaz FO, Zorzet A. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 18:318–327.
144. World Health Organization. 2017. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis.
145. Piddock LJV. 2019. The global antibiotic research and development partnership (GARDP): researching

and developing new antibiotics to meet global public health needs. Medchemcomm 10:1227–1230.
146. Kostyanev T, Bonten MJM, O'Brien S, Steel H, Ross S, François B, Tacconelli E, Winterhalter M, Stavenger RA, Karlén A, Harbarth S, Hackett J, Jafri HS, Vuong C, MacGowan A, Witschi A, Angyalosi G, Elborn JS, de Winter R, Goossens H. 2016. The Innovative Medicines Initiative's New Drugs for Bad Bugs programme: European public-private partnerships for the development of new strategies to tackle antibiotic resistance. J Antimicrob Chemother 71:290–295.

Chapter

2

Nonclonal emergence of colistin resistance associated with mutations in the BasRS two-component system in *Escherichia coli* bloodstream isolates

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Abstract

Infections by multidrug-resistant Gram-negative bacteria are increasingly common, prompting the renewed interest in the use of colistin. Colistin specifically targets Gram-negative bacteria by interacting with the anionic lipid A moieties of lipopolysaccharides, leading to membrane destabilization and cell death. Here, we aimed to uncover the mechanisms of colistin resistance in nine colistin-resistant *Escherichia coli* strains and one *E. albertii* strain. These were the only colistin-resistant strains out of 1140 bloodstream *Escherichia* isolates collected in a tertiary hospital over a ten-year period (2006 - 2015). Core-genome phylogenetic analysis showed that each patient was colonised by a unique strain, suggesting that colistin resistance was acquired independently in each strain. All colistin-resistant strains had lipid A that was modified with phosphoethanolamine. In addition, two *E. coli* strains had hepta-acylated lipid A species, containing an additional palmitate compared to the canonical hexa-acylated *E. coli* lipid A. One *E. coli* strain carried the mobile colistin resistance (*mcr*) gene *mcr-1.1* on an IncX4-type plasmid. Through construction of chromosomal transgene integration mutants, we experimentally determined that mutations in *basRS*, encoding a two-component signal transduction system, contributed to colistin resistance in four strains. We confirmed these observations by reversing the mutations in *basRS* to the sequences found in reference strains, resulting in loss of colistin resistance. While the *mcr* genes have become a widely studied mechanism of colistin resistance in *E. coli*, sequence variation in *basRS* is another, potentially more prevalent but relatively underexplored, cause of colistin resistance in this important nosocomial pathogen.

Importance

Multidrug resistance among Gram-negative bacteria has led to the use of colistin as a last-resort drug. The cationic colistin kills Gram-negative bacteria through electrostatic interaction with the anionic lipid A moiety of lipopolysaccharides. Due to increased use in clinical and agricultural settings, colistin resistance has recently started to emerge. In this study, we used a combination of whole genome sequence analysis and experimental validation to characterise the mechanisms through which *E. coli* strains from bloodstream infections can develop colistin resistance. We found no evidence of direct transfer of colistin-resistant isolates between patients. The lipid A of all isolates was modified by the addition of phosphoethanolamine. In four isolates, colistin resistance was experimentally verified to be caused by mutations in the *basRS* genes, encoding a two-component regulatory system. Our data show that chromosomal mutations are an important cause of colistin resistance among clinical *E. coli* isolates.

Introduction

Escherichia coli is a Gram-negative opportunistic pathogen that is a common cause of bloodstream, urinary tract, and enteric infections (1). The rising prevalence of antibiotic resistance in *E. coli*, in part due to the increasing global spread of the successful multidrug-resistant clade C lineage of ST131, may limit options for future treatments of infections (2, 3). Due to the emergence and spread of multidrug-resistant clones of *E. coli* and other Enterobacteriaceae, and the lack of new antibiotics targeting Gram-negative bacteria, colistin (polymyxin E) is increasingly used, despite its neuro- and nephrotoxic side effects, in the treatment of clinical infections with multidrug-resistant and carbapenem-resistant *E. coli* and other Enterobacteriaceae (4–6).

Colistin is a cationic, amphipathic molecule consisting of a non-ribosomal synthesized decapeptide and a lipid tail (7, 8). Colistin specifically targets Gram-negative bacteria by binding to the anionic phosphate groups of the lipid A moiety of lipopolysaccharides (LPS) through electrostatic interactions (7–9). Colistin destabilizes the outer membrane, but the subsequent disruption of the inner membrane ultimately leads to cell death (9, 10). Acquired colistin resistance has been reported in various Gram-negative bacteria that were isolated from clinical, veterinary, and environmental sources (11–13). The best-documented mechanism of colistin resistance involves the modification of lipid A with cationic groups to counteract the electrostatic interactions between colistin and lipid A (9). Lipid A modifications in Enterobacteriaceae may be mediated by the acquisition of mutations in chromosomally located genes or the acquisition of a mobile genetic element carrying one of the mobile colistin resistance (*mcr*) genes, which encode phosphoethanolamine transferases that catalyse the addition of a cationic phosphoethanolamine group to lipid A (14–16).

Among Enterobacteriaceae, colistin resistance has been most intensively studied in *Salmonella* and *Klebsiella pneumoniae* in which mutations in the regulatory genes *mgrB*, *phoPQ* and *pmrAB* are important mechanisms leading to resistance (15, 17–19). In *E. coli* however, mutations in *mgrB* and *phoPQ* have not been reported to lead to colistin resistance. This may be caused by the increased rate of dephosphorylation of PmrA (BasR in *E. coli*) by PmrB (BasS in *E. coli*) in *E. coli* compared to other Enterobacteriaceae, which effectively negates the possible activating effects of mutations in *phoPQ* or *mgrB*, through PmrD, on the levels of phosphorylated BasR. This may explain why not all of the previously described mutations reported to lead to colistin resistance in *Salmonella* and *Klebsiella* confer resistance in *E. coli* (14, 20–22). In addition, *phoPQ* expression in *E. coli* is not only controlled by MgrB but also by the sRNA MicA, adding to the mechanisms

controlling PhoPQ activation and making it less likely that the deletion or inactivation of *mgrB* can contribute to colistin resistance in *E. coli* (14, 23). This may explain why colistin resistance in clinical *E. coli* strains has only been linked to mutations in *basRS* (24–28), although experimental validation of the role of these mutations in colistin resistance is currently mostly lacking.

The PmrAB (BasRS) two-component system plays a crucial role in mediating the modification of LPS that lead to colistin resistance in Gram-negative bacteria (14, 17). Normally, this two-component system is activated by environmental stimuli, such as the presence of antimicrobial peptides or a low pH. Activation can increase virulence and survival through evasion of the host immune system by upregulating genes associated with modification of LPS, which is the predominant immunogenic molecule of Gram-negative bacteria (29, 30). In *E. coli*, the activation of BasRS leads to increased expression of various operons, including its own. This operon also includes *eptA*, which encodes a lipid A-specific phosphoethanolamine transferase (11, 14, 31).

Relatively little is known about colistin resistance mechanisms in *E. coli*, other than the acquisition of *mcr* genes (32). Therefore, we studied a collection of colistin-resistant *E. coli* strains from bloodstream infections by a combination of whole genome sequencing and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis of their lipid A, to identify colistin resistance mechanisms in *E. coli*. The role of mutations in *basRS* was investigated through the construction of chromosomal integration mutants of different *basRS* alleles.

Results

Low prevalence of colistin resistance in invasive *Escherichia* bloodstream isolates

A total of 1140 bloodstream isolates (collected from January 2006 to December 2015) for which species identification and automated antibiotic susceptibility testing had previously been performed, were available for this study. Twelve isolates were deemed resistant to colistin through routine diagnostic procedures. Two of those isolates were isolated from the same patient, on the same day, and were thus considered duplicates, and only one of these was included in this study. In ten of the eleven remaining isolates, colistin resistance, defined as an minimal inhibitory concentration (MIC) > 2 µg/ml colistin, was confirmed through broth microdilution (Table 1). Strain A783 was a false positive for colistin resistance during automated susceptibility testing in routine diagnostic procedures, and was excluded from subsequent analyses, leaving ten isolates for further investigation.

The estimated prevalence of colistin resistance in *E. coli* strains causing bloodstream infections isolated from January 2006 to December 2015 was thus determined to be 0.88%. Three patients had received colistin in the three months before isolation of the colistin-resistant strain (Table 1). Two of these patients received colistin to treat infections, but all three patients were also administered colistin as part of selective digestive or oropharyngeal decontamination (SDD/SOD), a prophylactic antibiotic treatment widely used in Dutch intensive care

Table 1: Colistin-resistant *Escherichia* strains isolated from bloodstream infections.[a]

Strain	Colistin MIC (µg/ml)[b]	MLST	Date of isolation	History of colistin use
I1121	16	131	22 April 2015	Yes; inhalation and oral
H2129	8	131	22 July 2014	No
G821	16	131	19 March 2013	No
F2745	4	73	2 November 2012	No
E3090	8	10	12 November 2011	No
E2372	4	59	25 August 2011	No
E650	8	162	11 March 2011	No
D2373	8	6901	20 October 2010	Yes; oral
A2361	8	5268	3 November 2007	No
Z821	4	167	2 April 2006	Yes; oral

[a] Overview of colistin-resistant bloodstream isolates, including the MIC of colistin, MLST type determined through whole genome sequencing, date of isolation, and information on the use of colistin three months before the isolation of the colistin-resistant isolate, and if applicable, route of administration.
[b] The MIC values represent the median of three independent replicate experiments performed in triplicate.

units (33). The ten colistin-resistant strains were analyzed further in this study to determine their relatedness and mechanism through which they had developed colistin resistance.

Colistin resistance was independently acquired by each individual bloodstream *E. coli* isolate

To assess the phylogenetic relationships between the colistin-resistant strains, a phylogenetic tree was generated based on the genome assemblies of the colistin-resistant strains and 210 publicly available complete genome sequences (Supplemental Table S1). Based on a core-genome alignment of 874 kbp, we did not observe direct transmission of colistin-resistant strains between patients (Figure 1A). Three colistin-resistant strains (strains I1121, H2129, and G821) belonged to the globally disseminated ST131 clone, and all three were dispersed throughout the multidrug-resistant clade C of ST131(Figure 1A, Figure 1B) (3, 34). This indicates that the ST131 strains in this study have independently acquired colistin resistance. Strain A2361 clustered among *E. albertii* (Figure 1A), although it had been typed as *E. coli* in routine diagnostic procedures.

By screening for acquired antibiotic resistance genes through ResFinder 3.2, we found that only strain E3090 carried the *mcr* gene *mcr-1.1* (0.086% of all bloodstream isolates; Figure 1C). After long-read sequencing and hybrid assembly, the *mcr-1.1*-gene in this strain appeared to be located as the sole antibiotic resistance gene on a 32.7 kbp IncX4-type plasmid. This *mcr-1.1* carrying IncX4-type plasmid from E3090 shares 99% identity to the previously reported *mcr-1.1* carrying IncX4-type plasmid pMcr-1_Msc (GenBank accession MK172815.1) harboured by *E. coli* isolated from patients in Russia (35), confirming the global dissemination of this plasmid (36). In all strains studied here, a variety of acquired resistance genes was observed (Figure 1C), reflecting the non-clonal nature of the colistin-resistant strains. The three colistin-resistant ST131 strains possessed different repertoires of acquired resistance genes, further excluding recent transmission between patients of the ST131 strains studied here. Strain F2745 and E2372 carried only one, and two resistance genes respectively, while the *E. albertii* strain A2361 did not possess any acquired resistance genes.

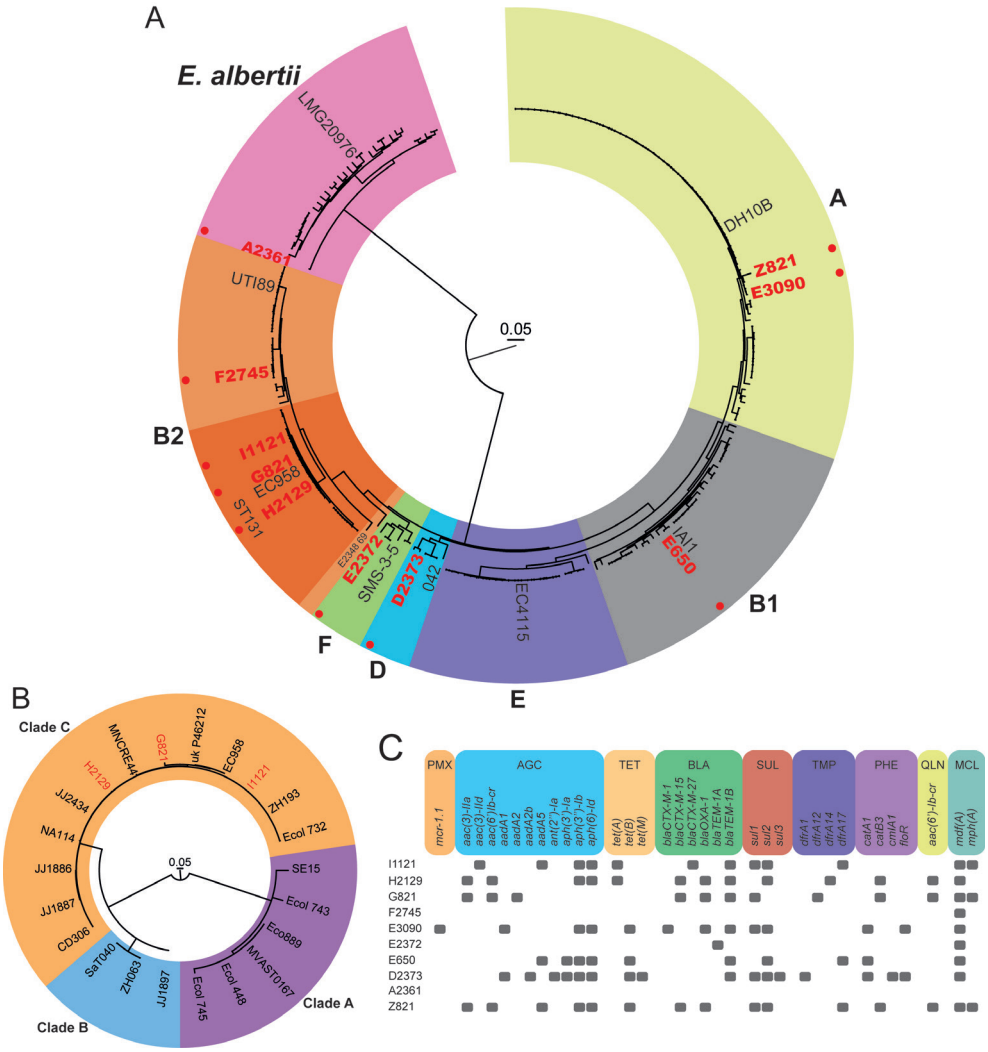

Figure 1: Colistin-resistant strains are not clonally related, and carry diverse acquired antibiotic resistance genes. A) The phylogenetic tree represents the core-genome alignment (874 kbp) of the colistin-resistant strains and 210 publicly available *E. coli* and *E. albertii* genome sequences. One representative reference strain per *E. coli* phylogroup is indicated (65). For *E. albertii*, the LMG20976 type strain is indicated (66). The different phylogroups of *E. coli* are indicated with coloured backgrounds. Phylogroup A is coloured yellow; B1, grey; B2, orange; D, blue; E, purple; F, green. The ST131 lineage of *E. coli* in phylogroup B2 is indicated by a dark orange background. The *E. albertii* branch is indicated by a pink background. The colistin-resistant strains characterized in this study are depicted in red, and highlighted by a red filled circle. **B)** The phylogenetic tree represents the core-genome alignment (3.55 Mbp) of the three colistin-resistant ST131 strains and 19 publicly available ST131 *E. coli* strains genome sequences. The colistin-resistant strains characterized in this study are depicted in red. Clades A, B, and C of ST131 are indicated by purple, blue, and orange backgrounds respectively. **C)** Antibiotic resistance genes in the genome sequences were detected by ResFinder 3.2 (56). Classes of antibiotic resistance genes are abbreviated as follow: PMX, polymyxin resistance; AGC, aminoglycoside resistance; TET, tetracycline resistance; BLA, ß-lactam resistance; SUL, sulfonamide resistance; TMP, trimethoprim resistance; PHE, phenicol resistance; QLN, quinolone resistance; MCL, macrolide.

Escherichia isolates exclusively acquire colistin resistance by modification of phosphate groups of lipid A

To determine which modifications to lipid A are affecting colistin resistance in *E. coli* we extracted lipid A from the clinical strains and the colistin-susceptible control *E. coli* strain MG1655, and subjected them to MALDI-TOF mass spectrometry. The lipid A produced by all *E. coli* strains showed lipid A species with a mass-to-charge ratio (*m/z*) of 1797 (Figure 2A), corresponding to the canonical unmodified *E. coli* hexa-acylated lipid A (Figure 2B). Colistin-resistant strains showed additional lipid A species at *m/z* 1921, consistent with the addition of phosphoethanolamine (*m/z* 124) to the hexa-acylated species. Additional species were detected in the lipid A produced by strains E650 and Z821. Species *m/z* 2036 indicated the addition of palmitate (*m/z* 239) to the hexa-acylated species *m/z* 1797, whereas species *m/z* 2160 was consistent with the addition of palmitate to the hexa-acylated lipid A species containing phosphoethanolamine (*m/z* 1910).

The *E. albertii* strain A2361 produced lipid A distinct from *E. coli*. Species *m/z* 1825 is likely to represent a hexa-acylated species corresponding to two glucosamines, two phosphates, four 3-OH-C_{14}, and two C_{14} (Figure 2C) Species *m/z* 1948 is consistent with the addition of phosphoethanolamine to the hexa-acylated species, with a further addition of palmitate to produce lipid A species *m/z* 2187. Species *m/z* 1868 and *m/z* 2107 could correspond to the loss of the second phosphate group, compared to *m/z* 1948, and *m/z* 2187.

Identification of mutations in *basRS* as candidate mutations involved in colistin resistance

Because chromosomal mutations in *basRS*, but not in other regulatory systems, have previously been suggested to cause colistin resistance in *E. coli* (24–28), we next aimed to establish the contribution of the *basRS* alleles in the colistin-resistant phenotype of these bloodstream isolates. Due to the multidrug-resistant nature of the clinical isolates (Figure 1C), we were unable to generate targeted mutations in these strains. Therefore, we made chromosomal transgene insertion mutants of the different *basRS* alleles in the *att*Tn*7* site in the BW25113-derived Δ*basRS* strain BW27848 using the Tn*7* transposon system. By making chromosomal transgenes insertions, rather than using an *in trans* complementation method, we excluded copy number effects by plasmids, and the need to use antibiotics to select for the presence of a plasmid used for *in trans* complementation. Since BW27848 still possesses the gene encoding for the phosphoethanolamine transferase EptA, we constructed sequences that consisted of the fused sequences of the promotor

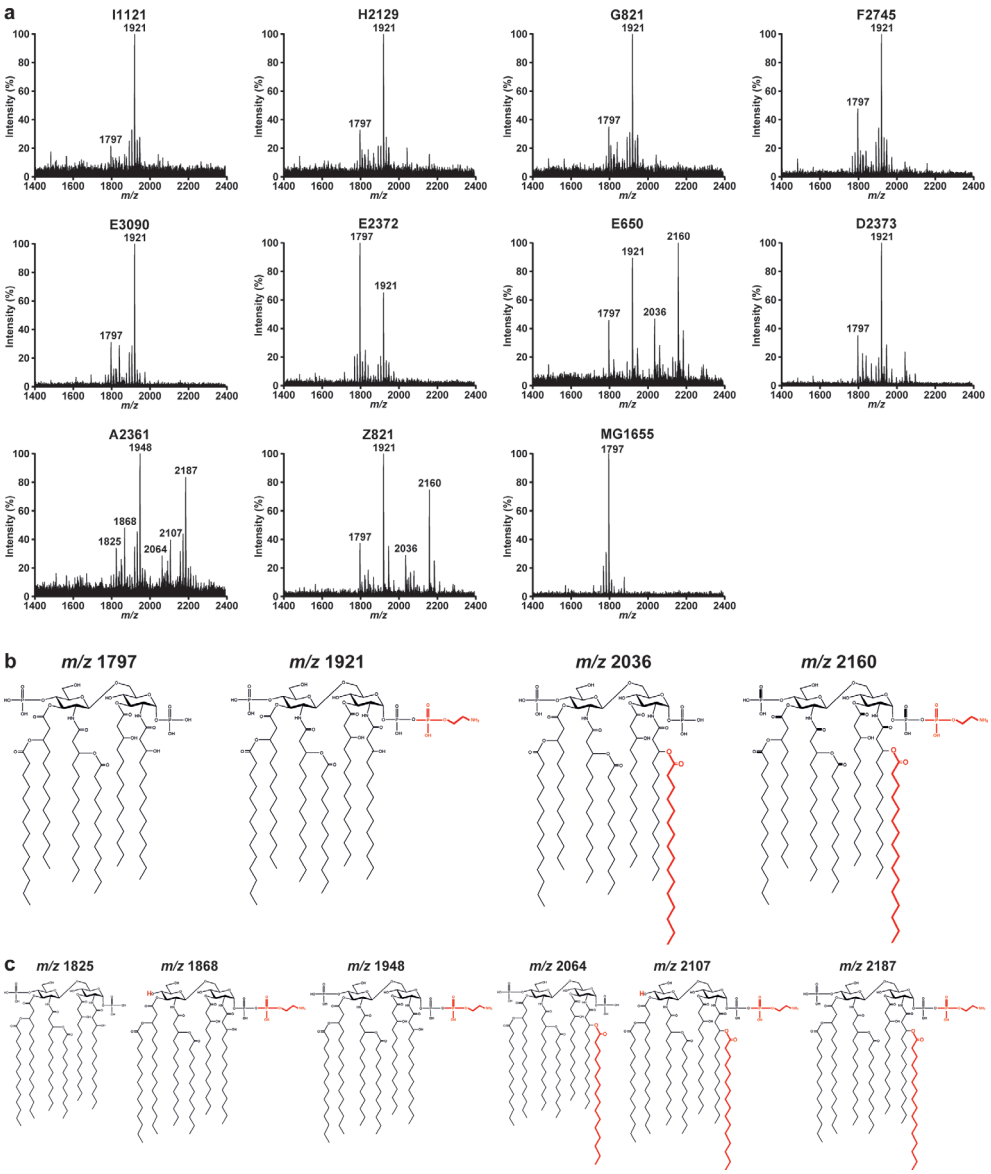

Figure 2: MALDI-TOF spectra of lipid A from colistin-resistant nosocomial *Escherichia* strains. Negative ion MALDI-TOF mass spectrometry spectra of lipid A purified from **A)** colistin-resistant strains and colistin-susceptible MG1655. Data represent the mass to charge (*m/z*) ratios of each lipid A species detected and are representative of three extractions. **B)** Proposed lipid A structures of the species produced by *E. coli* strains. **C)** Proposed lipid A structures of *E. albertii* strain A2361. Modifications to unmodified lipid A are depicted in red.

region of the *eptA-basR-basS* operon and the *basRS* coding sequences in order to prevent *eptA* gene dosage-dependent effects. We were unable to generate the construct for strain E650, presumably due to the toxicity of the insert.

The colistin MIC determination of the generated *basRS* chromosomal transgene insertion mutants from strains I1121, H2129, G821, and Z821 had higher colistin MIC values than the BW27848::Tn*7*-empty strain, with observed MIC values ≥16-fold higher than that of the BW27848::Tn*7*-empty strain (Table 2). As expected, the *basRS* allele of the *mcr-1.1* positive strain E3090 did not lead to colistin resistance. We were unable to show the contribution of *basRS* to colistin resistance in the additional four colistin-resistant strains (F2745, E2372, D2373, A2361) that lacked *mcr-1.1*.

Mutations in the *basRS* genes contribute to colistin resistance in *E. coli*

By construction of the chromosomal transgene insertion mutants, we identified the ability of the *basRS* sequences of four strains (I1121, H212, G821, and Z821) to cause colistin resistance in BW27848. To identify the mutations in the *basRS* alleles of these strains that contribute to resistance, we compared the *basRS* encoding sequences of those strains causing resistance to the phylogenetically most closely related publicly available *E. coli* genome sequences used in the construction of Figure 1A. None of these reference strains were reported to be colistin-resistant, or carried any of the *mcr* genes. This comparison revealed four distinct mutations: a L10R substitution in BasS in I1121, a G53S substitution in BasR in H2192, the

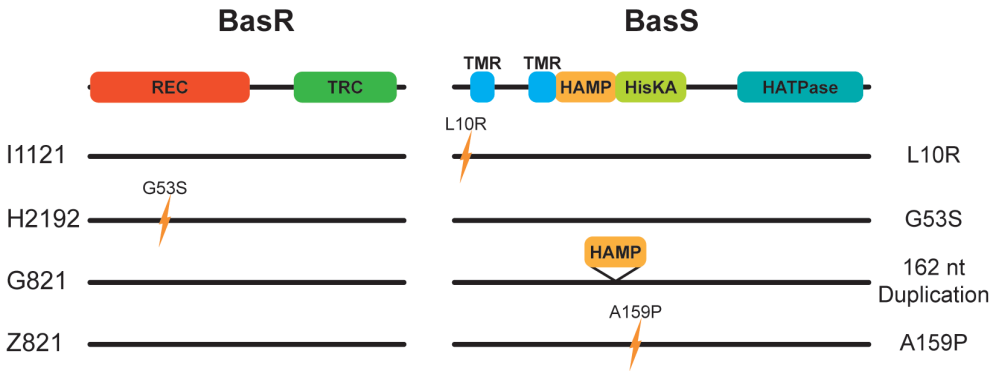

Figure 3: Conservation and prediction of functional effects of mutations in *basRS*. Comparison of the *basRS* sequences of colistin-resistant strains and publicly available genome sequences led to the identification of mutations in *basRS* that could have a role in colistin resistance. Domains of BasR and BasS were predicted using SMART (55). The domains are REC: CheY homologous receiver domain, TRC: Transcriptional regulatory protein, C terminal (Trans_reg_c), HAMP: Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases domain, HisKA: His Kinase A (phosphoacceptor) domain, HATPase: Histidine kinase like ATPases (HATPase_c). The two transmembrane regions (TMR) in BasS are highlighted in blue.

duplication of the HAMP domain in BasS in G821, and a A159P substitution in BasS in Z821 (Figure 3). As expected, in the *mcr-1.1* positive strain E3090 no mutations in *basRS* were identified.

We hypothesised that the observed mutations were impacting the normal functioning of the BasRS two-component system. To assess whether the mutations in *basRS* identified by comparing the *basRS* sequences of the clinical strains I1121, H2129, G821, and Z821, and their closest match in the set of 178 publicly available *E. coli* genome sequences (Figure 3) were causal to the development of colistin resistance, the identified mutations were reversed through site-directed inverse PCR mutagenesis to match the *basRS* alleles of the publicly available genome sequences. The MIC values of these mutants returned to levels similar to that of the colistin-susceptible BW27848::Tn*7*-empty strain (Table 2). These experiments support the involvement of *basRS* sequence variation in colistin resistance in *E. coli*.

Table 2. Colistin MICs of strains generated in this study.[a]

Strain	Colistin MIC (µg/ml)[b]
BW25113	0.25
BW27848	0.125
BW25113::Tn*7* Empty	0.25
BW27848::Tn*7* Empty	0.125
BW27848::Tn*7* BW25113	0.125
BW27848::Tn*7* I1121	2
BW27848::Tn*7* I1121m	0.25
BW27848::Tn*7* H2129	4
BW27848::Tn*7* H2129m	0.25
BW27848::Tn*7* G821	4
BW27848::Tn*7* G821m	0.25
BW27848::Tn*7* F2745	0.25
BW27848::Tn*7* E3090	0.125
BW27848::Tn*7* E2372	0.25
BW27848::Tn*7* D2373	0.5
BW27848::Tn*7* A2361	0.125
BW27848::Tn*7* Z821	2
BW27848::Tn*7* Z821m	0.125

[a] *E. coli* strain BW27848 is the Δ*basRS* mutant of BW25113 (49). The *basRS* alleles of colistin-resistant strains from this study were inserted into the *att*Tn*7* site of BW27848. The addition of "m" to a strain name indicates that the construct has been modified through inverse PCR site directed mutagenesis to reverse the mutation associated with colistin resistance.

[b] The values represent the median of three independent replicate experiments performed in triplicate.

Discussion

In the present study, we set out to characterise the mechanisms through which *E. coli* bloodstream isolates can develop colistin resistance through a combination of whole genome sequence analysis and experimental validation. We did not find evidence for transfer of colistin-resistant strains between patients, suggesting that colistin resistance has been acquired independently in all cases. In seven patients colistin-resistant strains were isolated without the patients being previously exposed to the drug. All colistin-resistant strains had LPS that was modified by the addition of phosphoethanolamine to the lipid A moiety of LPS. Resistance in one of the bloodstream isolates could be explained by the acquisition of *mcr-1.1*. In four other strains, we identified mutations in *basRS* that contribute to colistin resistance. Although colistin-susceptible strains that were isogenic to the resistant strains were not available, we were able to pinpoint the mutations in *basRS* leading to resistance in these strains by matching the genomic sequences of our nosocomial isolates with publicly available genomes, none of which were reported to be colistin-resistant, and subsequent construction of chromosomally integrated *basRS* transgene alleles in the Δ*basRS* strain BW27848. The mechanisms of colistin resistance in the remaining five strains remain to be characterized.

Some of the mutations we experimentally link to colistin resistance in this study, have previously been associated with colistin resistance or the functioning of the BasRS two-component system. In this study, we demonstrated that the amino acid change L10R in BasS (strain I1121) also confers colistin resistance. An amino acid substitution in the same position of BasS (L10P) was previously experimentally proven to cause colistin resistance in *E. coli* (26). The glycine in position 53 of BasR has previously been reported to be altered in colistin-resistant Enterobacteriaceae (37, 38) including in *E. coli* (39). The G53S change specifically, as in isolate H2192, has been experimentally proven to contribute to colistin resistance in *Klebsiella* (previously *Enterobacter*) *aerogenes* (40, 41) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (42) and we extend those findings to *E. coli* here. The previously unidentified duplication of 162 nucleotides in *basS* (strain G821) leads to the introduction of a second HAMP domain in BasS and confers colistin resistance in the BW27848 background. The HAMP domain is widespread in bacteria and is commonly involved in signal transduction as part of two-component systems (43). We hypothesise that the addition of an extra HAMP domain in BasS may change signal transduction in the protein, leading to the constitutive activation of the histidine kinase domain of BasS, increased phosphorylation of BasR and upregulated expression of *eptA*, ultimately resulting in the addition of phosphoethanolamine to lipid A. Finally, we demonstrate that the A159P substitution in BasS (observed in strain Z821) contributes to colistin

resistance. A mutation leading to a A159V substitution was found in an *in vitro* evolution study in which *E. coli* was evolved towards colistin resistance (44), and in clinical colistin-resistant *E. coli* isolates (45), but experimental confirmation of the role of alterations in A159 in colistin resistance in *E. coli* was so far lacking. Our data suggest that the *basRS* alleles of three *E. coli* strains (F2745, E2372, and D2373), and the *E. albertii* strain A2361, do not confer resistance in the BW25113 *E. coli* background. Because *E. albertii* is phylogenetically distinct from *E. coli*, its *basRS* allele may not function optimally in an *E. coli* background, explaining the inability of the transgene insertion complementation in the *basRS* deletion of BW25113 *E. coli* strain to cause colistin resistance (46). We are unable to explain the colistin resistance mechanisms of the clinical isolates F2745, E2372, and D2373. It is likely that these strains have become resistant to colistin through other mutations that finally lead to the modification of lipid A by phosphoethanolamine.

The observed modification of lipid A with phosphoethanolamine in all isolates underlines the crucial role of phosphoethanolamine transferases in the ability of *Escherichia* to become resistant to polymyxins (14). The lipid A of three of the colistin-resistant strains was also modified with palmitate, but the contribution of lipid A palmitoylation to colistin resistance in clinical *E. coli* strains is currently unknown. We did not observe modifications of lipid A by 4-amino-4-deoxy-L-arabinose in the colistin-resistant isolates. While this modification was shown to contribute to polymyxin B resistance under low Mg^{2+} conditions in a laboratory isolate of *E. coli* (20), it may be rare in clinical *E. coli* isolates. Indeed, Sato *et al.* also exclusively found phosphoethanolamine-modified lipid A in colistin-resistant clinical *E. coli* isolates (24). The reliance of *Escherichia* on the modification of lipid A by phosphoethanolamine to acquire colistin resistance, suggests that the inhibition of this class of enzymes by blocking the conserved catalytic site (31) could be a target for future drug development and opens the possibility of combination therapy with colistin and an inhibitor of phosphoethanolamine transferase (47). With the increasing clinical issues posed by infections with multidrug-resistant Gram-negative bacteria, there is an urgent need to better understand resistance mechanisms to last-resort antibiotics like colistin. While the discovery of the *mcr* genes have generated considerable interest in transferable colistin resistance genes, our data suggest that chromosomal mutations remain an important cause of colistin resistance among clinical isolates in the genus *Escherichia*.

Materials and methods

Ethical statement

Approval to obtain data from patient records was granted by the Medical Ethics Review Committee of the University Medical Center Utrecht, in Utrecht, the Netherlands (project numbers 16/641 and 18/472).

Colistin-resistant *E. coli* strains were isolated as part of routine diagnostic procedures. This aspect of the study did not require consent or ethical approval by an institutional review board.

Bacterial strains, growth conditions, and chemicals

Colistin-resistant *E. coli* strains from bloodstream infections were obtained retrospectively from the strain collection of the clinical microbiology laboratory of the University Medical Center Utrecht in Utrecht, the Netherlands. In initial routine diagnostic procedures, blood cultures were plated on TSA plates with 5% sheep blood. Strains collected up to 2011 were identified and their antibiogram was determined using the BD Phoenix automated identification and susceptibility testing system (Becton Dickinson, Vianen, the Netherlands). From 2011 onwards, species determination was performed by MALDI-TOF on a Bruker microflex system (Leiderdorp, the Netherlands). *E. coli* strain BW25113 and the BW25113-derived Δ*basRS* strain BW27848 from the Keio collection were obtained from the Coli Genetic Stock Center (48, 49). Strains were grown in Lysogeny Broth (LB; Oxoid, Landsmeer, the Netherlands) at 37°C with agitation at 300 rpm unless otherwise noted, with exception of strains containing pGRG36, which were grown at 30°C (50). When appropriate, kanamycin (50 µg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands), and ampicillin (100 µg/ml; Sigma-Aldrich) were used. Colistin sulphate was obtained from Duchefa Biochemie (Haarlem, the Netherlands). L-(+)-arabinose was obtained from Sigma-Aldrich. Plasmids were purified using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, Landsmeer, the Netherlands). PCR products were purified from gel using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific).

Determination of minimal inhibitory concentration

The MICs of colistin were determined as previously described (51), in line with the

recommendations of a joint working group of the Clinical & Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf), using BBL™ Mueller Hinton II (cation-adjusted) broth (MHCAB; Becton Dickinson), untreated Nunc 96-wells round bottom polystyrene plates (Thermo Scientific), and Breathe-Easy sealing membranes (Sigma-Aldrich). The breakpoint value of an MIC > 2 µg/ml for colistin resistance in *E. coli* was obtained from EUCAST.

Genomic DNA isolation and whole-genome sequencing

Genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega, Leiden, the Netherlands) according to the manufacturer's instructions. DNA concentrations of the genomic DNA preparations were measured with the Qubit dsDNA Broad Range Assay kit and the Qubit 2.0 fluorometer (Life Technologies, Bleiswijk, the Netherlands) and were all higher than 20 ng/µl.

Sequence libraries for Illumina sequencing were prepared using the Nextera XT kit (Illumina, San Diego, CA) according to the manufacturer's instructions with 1 ng genomic DNA as input. Libraries were sequenced on an Illumina MiSeq system with a 500-cycle (2 × 250 bp) MiSeq reagent kit v2.

For strain E3090, we performed long-read sequencing using the MinION platform (Oxford Nanopore Technologies) to fully resolve the *mcr-1.1* plasmid. MinION library preparation for barcoded 2D long-read sequencing was performed using the SQK-LSK208 kit (Oxford Nanopore Technologies, Oxford, England, United Kingdom), according to the manufacturer's instructions, with G-tube (Covaris, Woburn, Massachusetts, United States of America) shearing of 1 µg chromosomal DNA for 2 x 120 seconds at 1500 x *g*. Sequencing was performed on the MinION sequencer (Oxford Nanopore Technologies) using 2D barcoded sequencing through a SpotON Flow Cell Mk I (R9.4; Oxford Nanopore Technologies).

Genome assembly, MLST typing, and identification of antibiotic resistance genes

The quality of Illumina sequence data was assessed using FastQC v0.11.5 (https://github.com/s-andrews/FastQC). Raw Illumina sequencing reads were trimmed for quality using nesoni v0.115 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) using standard settings with

the exception of a minimum read length of 100 nucleotides. *De novo* genome assembly of the trimmed Illumina short-read data was performed using SPAdes v3.6.2 with the following settings: kmers used: 21, 33, 55, 77, 99, or 127, "careful" option turned on and cut-offs for final assemblies: minimum contig/scaffold size = 500 bp, and minimum contig/scaffold average Nt coverage = 10-fold (52).

MinION sequence read data in FastQ format was extracted from Metrichor base-called raw FAST5 read-files using Poretools (53). A hybrid assembly for strain E3090 was generated with trimmed Illumina short-read data and Oxford Nanopore Technologies MinION long-read data by using SPAdes v3.6.2 with the same settings as the Illumina short-read assemblies, and specifying the long-read data with the --nanopore flag.

Gene prediction and annotation was performed using Prokka (54), using standard settings. Protein domains were predicted using the SMART server (55). MLST typing was performed using the mlst package v2.10 (https://github.com/tseemann/mlst), using standard settings. Assembled contigs were assessed for antibiotic resistance genes using ResFinder 3.2 (56), using standard settings.

Core-genome phylogenetic analysis and determination of mutations in candidate colistin resistance determinants

Genome assemblies generated in this study with Illumina data were aligned with 178 complete *E. coli* genomes and 32 *E. albertii* genomes that were available from NCBI databases on 24 June 2016 (Supplemental Table S1) using Parsnp v1.2 (57). MEGA6 was used to midpoint root and visualize the phylogenetic tree (58). We identified whether non-synonymous mutations were present in *basRS* by pairwise comparison of the gene sequences of colistin-resistant isolates to their closest matching publicly available genome from the phylogenetic tree using BLAST (59). Mutations that were identified in the genome sequences were confirmed through PCR (oligonucleotide primer sequences are provided in Supplemental Table S2) and subsequent Sanger sequencing of the PCR product by Macrogen (Amsterdam, the Netherlands).

Isolation and analysis of lipid A

Isolation of lipid A molecules and subsequent analysis by negative-ion MALDI-TOF mass spectrometry was performed as previously described (19, 60, 61). Briefly, *Escherichia* strains were grown in LB (Oxoid) and the lipid A was purified from stationary cultures using the ammonium hydroxide/isobutyric acid method

described earlier (62). Mass spectrometry analyses were performed on a Bruker autoflex™ speed TOF/TOF mass spectrometer in negative reflective mode with delayed extraction using as matrix an equal volume of dihydroxybenzoic acid matrix (Sigma-Aldrich) dissolved in (1:2) acetonitrile-0.1% trifluoroacetic acid. The ion-accelerating voltage was set at 20 kV. Each spectrum was an average of 300 shots. A peptide calibration standard (Bruker) was used to calibrate the MALDI-TOF. Further calibration for lipid A analysis was performed externally using lipid A extracted from *E. coli* strain MG1655 grown in LB medium at 37°C.

Construction of chromosomal *basRS* transgene insertions

Chromosomal transgene insertions of *basRS* were constructed in BW27848 by utilizing the Tn*7* transposon system on the pGRG36 plasmid (50). The promoter of the *eptA-basRS* operon was fused to the *basRS* coding sequence by separate PCRs for the promotor region and the *basRS* amplicon, with high fidelity Phusion Green Hot Start II DNA Polymerase (Thermo Fisher Scientific) using strain-specific primers (Supplemental Table S2; oligonucleotides were obtained from Integrated DNA Technologies, Leuven, Belgium). The promoter and the *basRS* amplicon were subsequently fused by overlap PCR. Fused PCR products were cloned into pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR Cloning kit (Thermo Fisher Scientific), and subsequently subcloned into pGRG36 (50). Electrocompetent BW25113 and BW27848 *E. coli* cells were prepared as described previously (63) and transformed using the following settings: voltage 1800 V, capacitance 25 µF, resistance 200 Ω, with a 0.2 cm cuvette using the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Veenendaal, the Netherlands). Transformants were grown at 30°C. After confirming integration of the Tn*7* transposon at the *att*Tn*7* site by PCR (primers listed in Supplemental Table S2) and Sanger sequencing (Macrogen), the pGRG36 plasmid was cleared by culturing at 37°C.

Inverse PCR site-directed mutagenesis was performed on amplicons cloned in pCR-Blunt II-TOPO to reverse the mutations that were identified in colistin-resistant strains to the sequences of *basR* or *basS* in the closest matching publicly available genome (64). After gel purification of the amplified fragments, (hemi) methylated fragments were digested using DpnI (New England Biolabs (NEB), Ipswich, Massachusetts, United States of America). Subsequently, the vector was recircularized using the Rapid DNA Ligation kit (Thermo Fisher Scientific) after phosphorylation using T4 Polynucleotide kinase (NEB). The constructs were then transformed into chemically competent DH5α *E. coli* cells (Invitrogen, Landsmeer, the Netherlands). Mutated sequences were subsequently subcloned to pGRG36 as described above.

Data availability

Sequence data has been deposited in the European Nucleotide Archive (accession number PRJEB27030).

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A.B.J. conceived and designed experiments, performed experiments, analysed data, and wrote the manuscript. T.L.B. performed experiments, and analysed data. N.P.M. performed experiments, and analysed data. M.J.M.B. wrote the manuscript. R.J.L.W. wrote the manuscript. J.A.B. analysed data, and wrote the manuscript. W.v.S. conceived and designed experiments, wrote the manuscript, and supervised the study. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no conflicts of interest.

References

1. Kaper JB, Nataro JP, Mobley HLT. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140.
2. Kallonen T, Brodrick HJ, Harris SR, Corander J, Brown NM, Martin V, Peacock SJ, Parkhill J. 2017. Systematic longitudinal survey of invasive *Escherichia coli* in England demonstrates a stable population structure only transiently disturbed by the emergence of ST131. Genome Res 27:1437–1449.
3. McNally A, Kallonen T, Connor C, Abudahab K, Aanensen DM, Horner C, Peacock SJ, Parkhill J, Croucher NJ, Corander J. 2019. Diversification of colonization factors in a multidrug-resistant *Escherichia coli* lineage evolving under negative frequency-dependent selection. mBio 10:e00644-19.
4. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, Goossens H, Laxminarayan R. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci 115:E3463–E3470.
5. Lim LM, Ly N, Anderson D, Yang JC, Macander L, Jarkowski A, Forrest A, Bulitta JB, Tsuji BT. 2010. Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. Pharmacotherapy 30:1279–1291.
6. Nation RL, Li J. 2010. Colistin in the 21st Century. Curr Opin Infect Dis 22:535–543.
7. Domingues MM, Inácio RG, Raimundo JM, Martins M, Castanho MARB, Santos NC. 2012. Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. Biopolymers 98:338–344.
8. Landman D, Georgescu C, Martin DA, Quale J. 2008. Polymyxins revisited. Clin Microbiol Rev 21:449–465.
9. Velkov T, Thompson PE, Nation RL, Li J. 2010. Structure-activity relationships of polymyxin antibiotics. J Med Chem 53:1898–1916.
10. Sabnis A, Klöckner A, Becce M, Evans LE, Furniss RCD, Mavridou DAI, Stevens MM, Edwards AM. 2018. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. bioRxiv 479618v2.
11. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism Mcr-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168.
12. Halaby T, Kucukkose E, Janssen AB, Rogers MRC, Doorduijn DJ, van der Zanden AGM, al Naiemi N, Vandenbroucke-Grauls CMJE, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. Antimicrob Agents Chemother 60:6837–6843.
13. Islam A, Rahman Z, Monira S, Rahman MA, Camilli A, George CM, Ahmed N, Alam M. 2017. Colistin resistant *Escherichia coli* carrying *mcr-1* in urban sludge samples: Dhaka, Bangladesh. Gut Pathog 9:77.
14. Baron S, Hadjadj L, Rolain J-M, Olaitan AO. 2016. Molecular mechanisms of polymyxin resistance: knowns and unknowns. Int J Antimicrob Agents 48:583–591.
15. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev 30:557–596.
16. Zhang H, Srinivas S, Xu Y, Wei W, Feng Y. 2019. Genetic and biochemical mechanisms for bacterial lipid A modifiers associated with polymyxin resistance. Trends Biochem Sci 44:973–988.
17. Falagas ME, Rafailidis PI, Matthaiou DK. 2010. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Updat 13:132–138.
18. Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. Antimicrob Agents Chemother 57:5521–5526.
19. Kidd TJ, Mills G, Sá-Pessoa J, Dumigan A, Frank CG, Insua JL, Ingram R, Hobley L, Bengoechea JA. 2017. A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence. EMBO Mol Med 9:430–447.
20. Rubin EJ, Herrera CM, Crofts AA, Trent MS. 2015. PmrD is required for modifications to *Escherichia coli* endotoxin that promote antimicrobial resistance. Antimicrob Agents Chemother 59:2051–2061.
21. Winfield MD, Groisman EA. 2004. Phenotypic differences between *Salmonella* and *Escherichia coli* resulting from the disparate regulation of homologous genes. Proc Natl Acad Sci 101:17162–17167.
22. Chen HD, Jewett MW, Groisman EA. 2011. Ancestral genes can control the ability of horizontally acquired loci to confer new traits. PLoS Genet 7:e1002184.
23. Coornaert A, Lu A, Mandin P, Springer M, Gottesman S, Guillier M. 2010. MicA sRNA links the PhoP regulon to cell envelope stress. Mol Microbiol 76:467–479.

24. Sato T, Shiraishi T, Hiyama Y, Honda H, Shinagawa M, Usui M, Kuronuma K, Masumori N, Takahashi S, Tamura Y, Shin-ichi Y. 2018. Contribution of novel amino alterations in PmrA or PmrB to colistin resistance in *mcr*-negative *Escherichia coli* clinical isolates, including major multidrug-resistant lineages O25b:H4-ST131-H30Rx and non-x. Antimicrob Agents Chemother 62:e00864-18.
25. Froelich JM, Tran K, Wall D. 2006. A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. J Bacteriol 188:1180–1183.
26. Cannatelli A, Giani T, Aiezza N, Pilato V Di, Principe L, Luzzaro F, Galeotti CL, Rossolini GM. 2017. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. Sci Rep 7:5071.
27. Delannoy S, Le Devendec L, Jouy E, Fach P, Drider D, Kempf I. 2017. Characterization of colistin-resistant *Escherichia coli* isolated from diseased pigs in France. Front Microbiol 8:2278.
28. Bourrel AS, Poirel L, Royer G, Vuillemin X, Kieffer N, Clermont O, Denamur E, Nordmann P, Decousser J. 2019. Colistin resistance in Parisian inpatient faecal *Escherichia coli* as the result of two distinct evolutionary pathways. J Antimicrob Chemother 74:1521–1530.
29. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458:1191–1196.
30. Maeshima N, Fernandez RC. 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol 3:3.
31. Xu Y, Wei W, Lei S, Lin J, Srinivas S, Feng Y. 2018. An evolutionarily conserved mechanism for intrinsic and transferable polymyxin resistance. mBio 9:e02317-17.
32. Luo Q, Yu W, Zhou K, Guo L, Shen P, Lu H, Huang C, Xu H, Xu S, Xiao Y, Li L. 2017. Molecular epidemiology and colistin resistant mechanism of *mcr*-positive and *mcr*-negative clinical isolated *Escherichia coli*. Front Microbiol 8:2262.
33. Oostdijk EAN, Kesecioglu J, Schultz MJ, Visser CE, De Jonge E, Van Essen EHR, Bernards AT, Purmer I, Brimicombe R, Bergmans D, Van Tiel F, Bosch FH, Mascini E, Van Griethuysen A, Bindels A, Jansz A, Van Steveninck FAL, Van Der Zwet WC, Fijen JW, Thijsen S, De Jong R, Oudbier J, Raben A, Van Der Vorm E, Koeman M, Rothbarth P, Rijkeboer A, Gruteke P, Hart-Sweet H, Peerbooms P, Winsser LJ, Van Elsacker-Niele AMW, Demmendaal K, Brandenburg A, De Smet AMGA, Bonten MJM. 2014. Effects of decontamination of the oropharynx and intestinal tract on antibiotic resistance in ICUs a randomized clinical trial. J Am Med Assoc 312:1429–1437.
34. Ben Zakour NL, Alsheikh-Hussain AS, Ashcroft MM, Khanh Nhu NT, Roberts LW, Stanton-Cook M, Schembri MA, Beatson SA. 2016. Sequential acquisition of virulence and fluoroquinolone resistance has shaped the evolution of *Escherichia coli* ST131. mBio 7:e00347-16.
35. Ageevets V, Lazareva I, Mrugova T, Gostev V, Lobzin Y, Sidorenko S. 2019. IncX4 plasmids harbouring *mcr-1* genes: further dissemination. J Glob Antimicrob Resist 18:166–167.
36. Wang R, van Dorp L, Shaw LP, Bradley P, Wang Q, Wang X, Jin L, Zhang Q, Liu Y, Rieux A, Dorai-Schneiders T, Weinert LA, Iqbal Z, Didelot X, Wang H, Balloux F. 2018. The global distribution and spread of the mobilized colistin resistance gene *mcr-1*. Nat Commun 9:1179.
37. Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in Enterobacteriaceae. Emerg Infect Dis 22:1038–1043.
38. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Chaisiri K, Komalamisra C, Adelowo OO, Fagade OE, Banjo OA, Oke AJ, Adler A, Assous MV, Morand S, Raoult D, Rolain JM. 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. Int J Antimicrob Agents 44:500–507.
39. Trent MS, Ribeiro AA, Doerrler WT, Lin S, Cotter RJ, Raetz CRH. 2001. Accumulation of a polyisoprene-linked amino sugar in polymyxin-resistant *Salmonella typhimurium* and *Escherichia coli*: structural characterization and transfer to lipid A in the periplasm. J Biol Chem 276:43132–43144.
40. Diene SM, Merhej V, Henry M, El Filali A, Roux V, Robert C, Azza S, Gavory F, Barbe V, La Scola B, Raoult D, Rolain JM. 2013. The rhizome of the multidrug-resistant *Enterobacter aerogenes* genome reveals how new "killer bugs" are created because of a sympatric lifestyle. Mol Biol Evol 30:369–383.
41. Tindall BJ, Sutton G, Garrity GM. 2017. *Enterobacter aerogenes* Hormaeche and Edwards 1960 (Approved lists 1980) and *Klebsiella mobilis* Bascomb *et al.* 1971 (approved lists 1980) share the same nomenclatural type (ATCC 13048) on the approved lists and are homotypic synonyms, with consequences for the name *Klebsiella mobilis* Basacomb *et al.* 1971 (Approved Lists 1980). Int J Syst Evol Microbiol 67:502–504.

42. Sun S, Negrea A, Rhen M, Andersson DI. 2009. Genetic analysis of colistin resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother 53:2298–2305.
43. Schultz JE, Kanchan K, Ziegler M. 2015. Intraprotein signal transduction by HAMP domains: a balancing act. Int J Med Microbiol 305:243–251.
44. Lee J-Y, Choi M-J, Choi HJ, Ko KS. 2015. Preservation of acquired colistin resistance in Gram-negative bacteria. Antimicrob Agents Chemother 60:609–612.
45. Olaitan AO, Morand S, Rolain JM. 2016. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. Int J Antimicrob Agents 47:1–3.
46. van der Putten BC, Maramoros S, COMBAT consortium, Schultz C. 2019. Genomic evidence for revising the *Escherichia* genus and description of *Escherichia ruysiae* sp. nov. bioRxiv 781724v1.
47. Son SJ, Huang R, Squire CJ, Leung IKH. 2018. Mcr-1: a promising target for structure-based design of inhibitors to tackle polymyxin resistance. Drug Discov Today 24:206–216.
48. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006.0008.
49. Zhou L, Lei X, Bochner BR, Wanner BL. 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J Bacteriol 185:4956–4972.
50. McKenzie GJ, Craig NL. 2006. Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn*7* without need for selection of the insertion event. BMC Microbiol 6:39.
51. Andrews JM. 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48:5–16.
52. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin A V, Sirotkin A V, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477.
53. Loman NJ, Quinlan AR. 2014. Poretools: a toolkit for analyzing nanopore sequence data. Bioinformatics 30:3399–3401.
54. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.
55. Letunic I, Bork P. 2018. 20 years of the SMART protein domain annotation resource. Nucleic Acids Res 46:D493–D496.
56. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644.
57. Treangen TJ, Ondov BD, Koren S, Phillippy AM. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 15:524.
58. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729.
59. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic Local Alignment Search Tool. J Mol Biol 215:403–410.
60. Llobet E, Martínez-Moliner V, Moranta D, Dahlström KM, Regueiro V, Tomás A, Cano V, Pérez-Gutiérrez C, Frank CG, Fernández-Carrasco H, Insua JL, Salminen TA, Garmendia J, Bengoechea JA. 2015. Deciphering tissue-induced *Klebsiella pneumoniae* lipid A structure. Proc Natl Acad Sci U S A 112:E6369–E6378.
61. Llobet E, Campos MA, Giménez P, Moranta D, Bengoechea JA. 2011. Analysis of the networks controlling the antimicrobial-peptide-dependent induction of *Klebsiella pneumoniae* virulence factors. Infect Immun 79:3718–3732.
62. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res 46:1773–1778.
63. Sharma RC, Schimke RT. 1996. Preparation of electro-competent *E. coli* using salt-free growth medium. Biotechniques 20:42–44.
64. Silva D, Santos G, Barroca M, Collins T. 2017. Inverse PCR for point mutation introduction, p. 87–100. In Methods in Molecular Biology. Humana Press Inc.
65. McNally A, Cheng L, Harris SR, Corander J. 2013. The evolutionary path to extraintestinal pathogenic, drug-resistant *Escherichia coli* is marked by drastic reduction in detectable recombination within the core genome. Genome Biol Evol 5:699–710.
66. Huys G, Cnockaert M, Janda JM, Swings J. 2003. *Escherichia albertii* sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. Int J Syst Evol Microbiol 53:807–810.

Supplemental Materials

Supplemental Table S1: Strains and NCBI accession numbers of strains used in phylogenetic analysis.[a]

Strain[b]	MLST[c]	GenBank assembly accession	Organism	Closest match[d]
42	414	GCA_000027125.1	*Escherichia coli*	
536	127	GCA_000013305.1	*Escherichia coli*	
789	88	GCA_000819645.1	*Escherichia coli*	
1303	10	GCA_000829985.1	*Escherichia coli*	
6409	10	GCA_000814145.2	*Escherichia coli*	E3090
11128	16	GCA_000010765.1	*Escherichia coli*	
11368	21	GCA_000091005.1	*Escherichia coli*	
12009	17	GCA_000010745.1	*Escherichia coli*	
55989	3762	GCA_000026245.1	*Escherichia coli*	
180PT54	5638	GCA_001650275.1	*Escherichia coli*	
2009C3133	117	GCA_001420955.1	*Escherichia coli*	
2009EL2050	678	GCA_000299255.1	*Escherichia coli*	
2009EL2071	678	GCA_000299475.1	*Escherichia coli*	
2011C3493	678	GCA_000299455.1	*Escherichia coli*	
2011C3911	1727	GCA_001644725.1	*Escherichia coli*	
2012C4227	119	GCA_001420935.1	*Escherichia coli*	
2013C4465	335	GCA_001644745.1	*Escherichia coli*	
28RC1	11	GCA_001612475.1	*Escherichia coli*	
644PT8	ND	GCA_001650295.1	*Escherichia coli*	
943024	672	GCA_000801185.2	*Escherichia coli*	
ABU 83972	73	GCA_000148365.1	*Escherichia coli*	
ACN001	23	GCA_001051135.1	*Escherichia coli*	
ACN002	23	GCA_001515725.1	*Escherichia coli*	
APEC IMT5155	ND	GCA_000813165.1	*Escherichia coli*	
APEC O1	95	GCA_000014845.1	*Escherichia coli*	
APEC O78	23	GCA_000332755.1	*Escherichia coli*	
ATCC 25922	73	GCA_000743255.1	*Escherichia coli*	F2745
ATCC 8739	3021	GCA_000019385.1	*Escherichia coli*	
B7A	ND	GCA_000725265.1	*Escherichia coli*	
BL21 (TaKaRa)	93	GCA_000833145.1	*Escherichia coli*	
BL21(DE3) #1	93	GCA_000009565.2	*Escherichia coli*	
BL21(DE3) #2	93	GCA_000022665.2	*Escherichia coli*	
BL21Gold(DE3)pLysS AG	93	GCA_000023665.1	*Escherichia coli*	
C22711	678	GCA_000986765.1	*Escherichia coli*	
C2566	93	GCA_001559615.1	*Escherichia coli*	
C3026	10	GCA_001559675.1	*Escherichia coli*	
C3029	93	GCA_001559635.1	*Escherichia coli*	

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C321.deltaA	10	GCA_000474035.1	*Escherichia coli*	
C41(DE3)	93	GCA_000830035.1	*Escherichia coli*	
C43(DE3)	93	GCA_001039415.1	*Escherichia coli*	
CB9615	335	GCA_000025165.1	*Escherichia coli*	
CD306	131	GCA_001513615.1	*Escherichia coli*	
CE10	62	GCA_000227625.1	*Escherichia coli*	
CFSAN029787	99	GCA_001007915.1	*Escherichia coli*	
CFT073	73	GCA_000007445.1	*Escherichia coli*	
CI5	5082	GCA_000971615.1	*Escherichia coli*	
clone D i14	73	GCA_000233895.1	*Escherichia coli*	
clone D i2	73	GCA_000233875.1	*Escherichia coli*	
CQSW20	1060	GCA_001455385.1	*Escherichia coli*	
DH1 #1	1060	GCA_000023365.1	*Escherichia coli*	
DH1 #2	1060	GCA_000270105.1	*Escherichia coli*	
DH1Ec095	1060	GCA_001183645.1	*Escherichia coli*	
DH1Ec104	1060	GCA_001183665.1	*Escherichia coli*	
DH1Ec169	1060	GCA_001183685.1	*Escherichia coli*	
DHB4	10	GCA_001559655.1	*Escherichia coli*	
E2348/69	15	GCA_000026545.1	*Escherichia coli*	
E24377A	1132	GCA_000017745.1	*Escherichia coli*	
EC4115	11	GCA_000021125.1	*Escherichia coli*	
EC958	131	GCA_000285655.3	*Escherichia coli*	**G821 / H2129**
ECC1470	847	GCA_000831565.1	*Escherichia coli*	
Eco889	131	GCA_001663475.1	*Escherichia coli*	
Ecol_448	131	GCA_001618365.1	*Escherichia coli*	
Ecol_732	131	GCA_001617565.1	*Escherichia coli*	**I1121**
Ecol_743	131	GCA_001618325.1	*Escherichia coli*	
Ecol_745	131	GCA_001618345.1	*Escherichia coli*	
ECONIH1	648	GCA_000784925.1	*Escherichia coli*	
EDL933	11	GCA_000732965.1	*Escherichia coli*	
ER1821R	10	GCA_001663075.1	*Escherichia coli*	
ER2796	10	GCA_000800215.1	*Escherichia coli*	
ER3413	10	GCA_000800765.1	*Escherichia coli*	
ER3435	ND	GCA_000974885.1	*Escherichia coli*	
ER3440	ND	GCA_000974465.1	*Escherichia coli*	
ER3445	ND	GCA_000974535.1	*Escherichia coli*	
ER3446	ND	GCA_000974825.1	*Escherichia coli*	
ER3454	ND	GCA_000974405.1	*Escherichia coli*	
ER3466	ND	GCA_000974575.1	*Escherichia coli*	

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ER3475	ND	GCA_000974865.1	*Escherichia coli*	
ER3476	ND	GCA_000974505.1	*Escherichia coli*	
ETEC H10407	48	GCA_000210475.1	*Escherichia coli*	
FRIK2069	11	GCA_001651925.1	*Escherichia coli*	
FRIK2455	11	GCA_001651965.1	*Escherichia coli*	
FRIK2533	11	GCA_001651945.1	*Escherichia coli*	
G749	131	GCA_001566635.1	*Escherichia coli*	
HS	46	GCA_000017765.1	*Escherichia coli*	
HUSEC2011	678	GCA_000967155.1	*Escherichia coli*	
IAI1	1128	GCA_000026265.1	*Escherichia coli*	E650
IAI39	62	GCA_000026345.1	*Escherichia coli*	
IHE3034	95	GCA_000025745.1	*Escherichia coli*	
JEONG1266	11	GCA_001558995.2	*Escherichia coli*	
JJ1886	131	GCA_000493755.1	*Escherichia coli*	
JJ1887	131	GCA_001593565.1	*Escherichia coli*	
JJ1897	131	GCA_001513655.1	*Escherichia coli*	
JJ2434	131	GCA_001513635.1	*Escherichia coli*	
JW54371 substr. MG1655	10	GCA_001566335.1	*Escherichia coli*	
AG100	7415	GCA_000981485.1	*Escherichia coli*	
BW25113	1996	GCA_000750555.1	*Escherichia coli*	
BW2952	5967	GCA_000022345.1	*Escherichia coli*	
DH10B	4638	GCA_000019425.1	*Escherichia coli*	
GM4792 #1	10	GCA_001020945.2	*Escherichia coli*	
GM4792 #2	10	GCA_001021005.2	*Escherichia coli*	
HMS174	10	GCA_000953515.1	*Escherichia coli*	
MC4100	10	GCA_000499485.1	*Escherichia coli*	
MDS42	1060	GCA_000350185.1	*Escherichia coli*	
MG1655 #1	10	GCA_000005845.2	*Escherichia coli*	
MG1655 #2	10	GCA_000801205.1	*Escherichia coli*	
MG1655 #3	1060	GCA_001308065.1	*Escherichia coli*	
MG1655 #4	10	GCA_001544635.1	*Escherichia coli*	
MG1655_TMP32XR1	10	GCA_001308125.1	*Escherichia coli*	
MG1655_TMP32XR2	10	GCA_001308165.1	*Escherichia coli*	
RV308	10	GCA_000952955.1	*Escherichia coli*	
W3110	10	GCA_000010245.1	*Escherichia coli*	
KLY	10	GCA_000725305.1	*Escherichia coli*	
KO11	1079	GCA_000147855.3	*Escherichia coli*	
KO11FL	1079	GCA_000258025.1	*Escherichia coli*	
LF82	135	GCA_000284495.1	*Escherichia coli*	

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LY180	1079	GCA_000468515.1	*Escherichia coli*	
MNCRE44	131	GCA_000931565.1	*Escherichia coli*	
MRE600	ND	GCA_001542675.2	*Escherichia coli*	
MVAST0167	131	GCA_001566655.1	*Escherichia coli*	
NA114	131	GCA_000214765.2	*Escherichia coli*	
NCM3722	10	GCA_001043215.1	*Escherichia coli*	
NGF1	998	GCA_001660585.1	*Escherichia coli*	
Nissle 1917	73	GCA_000714595.1	*Escherichia coli*	
NRG 857C	135	GCA_000183345.1	*Escherichia coli*	
P12b	10	GCA_000257275.1	*Escherichia coli*	
PCN033	5147	GCA_000219515.3	*Escherichia coli*	D2373
PCN061	46	GCA_001029125.1	*Escherichia coli*	
REL606	93	GCA_000017985.1	*Escherichia coli*	
RM12579	335	GCA_000245515.1	*Escherichia coli*	
RM12581	32	GCA_000671295.1	*Escherichia coli*	
RM12761	6130	GCA_000662395.1	*Escherichia coli*	
RM13514	32	GCA_000520035.1	*Escherichia coli*	
RM13516	6130	GCA_000520055.1	*Escherichia coli*	
RM9387	2773	GCA_000801165.1	*Escherichia coli*	
RR1	10	GCA_001276585.1	*Escherichia coli*	
RS218	ND	GCA_000800845.2	*Escherichia coli*	
S51	7060	GCA_001660565.1	*Escherichia coli*	
S88	95	GCA_000026285.1	*Escherichia coli*	
Sakai	11	GCA_000008865.1	*Escherichia coli*	
Sanji	167	GCA_001610755.1	*Escherichia coli*	**Z821**
Santai	1011	GCA_000827105.1	*Escherichia coli*	
SaT040	131	GCA_001566615.1	*Escherichia coli*	
SE11	156	GCA_000010385.1	*Escherichia coli*	
SE15	131	GCA_000010485.1	*Escherichia coli*	
SEC470	48	GCA_000987875.1	*Escherichia coli*	
SF088	95	GCA_001280325.1	*Escherichia coli*	
SF166	95	GCA_001280385.1	*Escherichia coli*	
SF173	95	GCA_001280405.1	*Escherichia coli*	
SF468	95	GCA_001280345.1	*Escherichia coli*	
SMS35	354	GCA_000019645.1	*Escherichia coli*	E2372
SQ110	10	GCA_000988425.1	*Escherichia coli*	
SQ171	10	GCA_000988445.1	*Escherichia coli*	
SQ2203	10	GCA_000988465.1	*Escherichia coli*	
SQ37	10	GCA_000988355.1	*Escherichia coli*	

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SQ88	10	GCA_000988385.1	*Escherichia coli*
SRCC 1675	11	GCA_001612495.1	*Escherichia coli*
SS17	11	GCA_000730345.1	*Escherichia coli*
SS52	11	GCA_000803705.1	*Escherichia coli*
ST2747 #1	6131	GCA_000599665.1	*Escherichia coli*
ST2747 #2	6131	GCA_000599685.1	*Escherichia coli*
ST2747 #3	6131	GCA_000599705.1	*Escherichia coli*
ST540 #1	540	GCA_000597845.1	*Escherichia coli*
ST540 #2	540	GCA_000599625.1	*Escherichia coli*
ST540 #3	540	GCA_000599645.1	*Escherichia coli*
ST648	648	GCA_001485455.1	*Escherichia coli*
TW14359	11	GCA_000022225.1	*Escherichia coli*
uk_P46212	131	GCA_001469815.1	*Escherichia coli*
UM146	643	GCA_000148605.1	*Escherichia coli*
UMNK88	100	GCA_000212715.2	*Escherichia coli*
UTI89	95	GCA_000013265.1	*Escherichia coli*
VR50	10	GCA_000968515.1	*Escherichia coli*
W #1	1079	GCA_000184185.1	*Escherichia coli*
W #2	1079	GCA_000258145.1	*Escherichia coli*
WS4202	11	GCA_001307215.1	*Escherichia coli*
Xuzhou21	11	GCA_000262125.1	*Escherichia coli*
YD786	410	GCA_001442495.1	*Escherichia coli*
ZH063	131	GCA_001577325.1	*Escherichia coli*
ZH193	131	GCA_001566675.1	*Escherichia coli*
24	4633	GCA_001514575.1	*Escherichia albertii*
jun51	678	GCA_001514595.1	*Escherichia albertii*
94389	11	GCA_001514625.1	*Escherichia albertii*
20H38	6057	GCA_001514555.1	*Escherichia albertii*
CB10113	6054	GCA_001514825.1	*Escherichia albertii*
CB9791	6049	GCA_001514845.1	*Escherichia albertii*
E2675	2683	GCA_001514865.1	*Escherichia albertii*
EC03127	6052	GCA_001514885.1	*Escherichia albertii*
EC03195	4947	GCA_001514905.1	*Escherichia albertii*
EC05160	3762	GCA_001514925.1	*Escherichia albertii*
EC0544	6058	GCA_001514945.1	*Escherichia albertii*
EC0581	6055	GCA_001514965.1	*Escherichia albertii*
EC06170	ND	GCA_001549955.1	*Escherichia albertii*
HIPH08472	ND	GCA_001514985.1	*Escherichia albertii*
K7394	10	GCA_001515005.1	*Escherichia albertii*

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K7744	10	GCA_001515025.1	*Escherichia albertii*	
K7756	10	GCA_001515045.1	*Escherichia albertii*	A2361
KF1	10	GCA_000512125.1	*Escherichia albertii*	
KU20110014	3762	GCA_001515065.1	*Escherichia albertii*	
LMG20976	383	GCA_000759775.1	*Escherichia albertii*	
NIAH_Bird_13	3762	GCA_001514645.1	*Escherichia albertii*	
NIAH_Bird_16	6056	GCA_001514665.1	*Escherichia albertii*	
NIAH_Bird_2	4606	GCA_001514685.1	*Escherichia albertii*	
NIAH_Bird_23	6059	GCA_001514705.1	*Escherichia albertii*	
NIAH_Bird_24	4634	GCA_001514725.1	*Escherichia albertii*	
NIAH_Bird_25	5967	GCA_001514745.1	*Escherichia albertii*	
NIAH_Bird_26	ND	GCA_001514765.1	*Escherichia albertii*	
NIAH_Bird_5	4736	GCA_001514785.1	*Escherichia albertii*	
NIAH_Bird_8	2700	GCA_001514805.1	*Escherichia albertii*	
TW07627	383	GCA_000155105.1	*Escherichia albertii*	
TW08933	1763	GCA_000208425.2	*Escherichia albertii*	
TW15818	ND	GCA_000208505.2	*Escherichia albertii*	

[a] 178 *E. coli* and 32 *E. albertii* genome sequences were used to construct the phylogenetic tree (Figure 1A).
[b] When multiple GenBank assemblies had the same strain name, a numerical indicator was added.
[c] If *in silico* MLST typing was not possible, this is identified by ND (not determined).
[d] The closest match to the colistin-resistant strains isolated in this study is indicated. Bold font signifies strains for which we were able to link mutations in *basRS* to colistin resistance.

Supplemental Table S2: Oligonucleotide sequences.

Primer[a]	Sequence (5'3')	Reference
BW25113 *glmS* Up	ATA TTC AGT CAA TTA CAA ACA TTA	This study
BW25113 *glmS* Down	CGA TCT TCT ACA CCG TTC	This study
Tn*7*R	CAC AGC ATA ACT GGA CTG ATT TC	(S1)[b]
Tn*7*R inward	GAA ATC AGT CCA GTT ATG CTG TG	This study
Tn*7*L	ATT AGC TTA CGA CGC TAC ACC C	(S1)[b]
Tn*7*L inward	GGG TGT AGC GTC GTA AGC TAA T	This study
BasRS sequencing Fwd	AAA GCC CGT ATC CGC AC	This study
BasRS sequencing Rev	GAT CTC ACG CAT GAT GTG GC	This study
BasRS deletion BW27848 check Fwd	CAA ACG CAA CAC TAT TCA CAA GAC	This study
BasRS deletion BW27848 check Rev	ATC TCT GAC GCG CAT ACT CTC	This study
pGRG36 check with Tn*7*L	ATA TGC ACA GAT GAA AAC GGT G	This study
BasRS promoter Fwd	CTT CCT CTA CTG CAT CTG GG	This study
BasRS promoter E650 Fwd	CTT CCT CTA CTG CAT TTG GG	This study
BasRS promoter A2361 Fwd	TTT TCT CTA CTG CAT CTG GG	This study
BasRS promoter Rev	CAC GGT GTT TCC ATC GA	This study
BasRS promoter A2361 Rev	CAC GGT ATT TCC ATC AA	This study
BasRS genes Fwd	ATG AAA ATT CTG ATT GTT GAA GA	This study
BasRS genes Rev	GTT CAG CGT GCT GGT GGT	This study
BasRS genes A2361 Rev	ATT CAG CGT GCT GGT CGT	This study
BasRS genes overlap Fwd	TGC GCA CTT TGT TCG ATG GAA ACA CCG TGA TGA AAA TTC TGA TTG TTG AAG ACG AT	This study
BasRS genes overlap A2361 Fwd	TGC GCA CTT TGT TTG ATG GAA ATA CCG TGA TGA AAA TTC TGA TTG TTG AAG ACG AT	This study
BasRS promoter NotI Fwd	TAT CCT GCG GCC GCC TTC CTC TAC TGC ATC TGG G	This study
BasRS promoter NotI E650 Fwd	TAT CCT GCG GCC GCC TTC CTC TAC TGC ATT TGG G	This study
BasRS promoter NotI A2361 Fwd	TAT CCT GCG GCC GCT TTT CTC TAC TGC ATC TGG G	This study

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BasRS genes XhoI Rev	TAT CCC CTC GAG GTT CAG CGT GCT GGT GGT	This study
BasRS genes XhoI A2361 Rev	TAT CCT CTC GAG ATT CAG CGT GCT GGT CGT	This study
BasRS promotor NotI short Fwd	TAT CCT GCG GCC GCC	This study
BasRS promotor NotI short A2361 Fwd	TAT CCT GCG GCC GCT	This study
BasRS promotor XhoI short Rev	TAT CCC CTC GAG GTT CA	This study
BasRS promotor XhoI short A2361 Rev	TAT CCT CTC GAG GTT CA	This study
I1121 BasS R10L mutagenesis Fwd	CGA CCA ATA TCG CTG CGC CAA CGG CTG	This study
I1121 BasS R10L mutagenesis Rev	GCG CAG AAA ACG CAT CAG ATT CAA TTA G	This study
H2129 BasR S53G mutagenesis Fwd	AGC CTG GTG GTA CTG GAT TTA GGC TTA CCC GAT G	This study
H2129 BasR S53G mutagenesis Rev	GTA ATG ACC GGC TTC AAG GCT TTG TTC CGC	This study
G821 BasS duplication mutagenesis Fwd[c]	TTT CAT TAT CGA GCG TGC TGG	This study
G821 BasS duplication mutagenesis Rev[c]	GGT TGT TTA CCG CTG ACG TC	This study
G821 BasS duplication check Fwd	ATC TGC TAT CAG GCG GTA CG	This study
G821 BasS duplication check Rev	GTT CGT CAT ACG AGG GGA GA	This study
Z821 BasS P159A mutagenesis Fwd	GAA CGC CAC TGG CGG GGG TGC GT	This study
Z821 BasS P159A mutagenesis Rev	GCA GTT CGT GCG CGA CGT CAG CGG TAA ACA A	This study

[a] Primers were used for all colistin-resistant strains unless a specific strain name is provided in the description. In the primers used for inverse PCR site-directed mutagenesis, the forward (Fwd) primer carries the desired mutation, whilst the reverse (Rev) primers complements the primer set for inverse PCR.
[b] S1. Choi K-H, Schweizer HP. 2006. mini-Tn*7* insertion in bacteria with single *att*Tn*7* sites: example *Pseudomonas aeruginosa*. Nat Protoc 1:153–161.
[c] The mutagenesis primers for strain G821 are designed to edit out the 162 nucleotide duplication in *basS*.

Chapter

3

Microevolution of acquired colistin resistance in Enterobacteriaceae isolated from ICU patients receiving selective decontamination of the digestive tract

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Abstract

Colistin is an antibiotic that targets the lipopolysaccharides present in Gram-negative bacteria. It is used as last-resort drug to treat infections with multidrug-resistant strains. Colistin is also used in selective decontamination of the digestive tract (SDD). SDD is a widely used treatment in the Netherlands, and prevents infections in patients admitted to an intensive care unit by selective eradication of opportunistic pathogens in the oropharyngeal and gut microbiota. In this study, we aimed to unravel the mechanisms of acquired colistin resistance in Gram-negative opportunistic pathogens obtained from SDD-treated patients through rectal swabs.

Through surveillance cultures of 428 patients, we obtained thirteen strains with acquired colistin resistance (*Escherichia coli,* n=9; *Klebsiella aerogenes*, n=3; *Enterobacter asburiae*, n=1) from five SDD-treated patients. Genome sequence analysis showed that there were multiple distinct colistin-resistant clones, but that they were clonally related within the same patient. In the *E. coli* strains, we observed a novel variant of *basR* encoding an 18 bp deletion, and a G19E substitution in BasS. We experimentally confirmed these variants to contribute to reduced colistin susceptibility. We also found previously well-described mechanisms that lead to colistin resistance (G53 substitution in BasR, and the acquisition of an *mcr-1.1* gene). In a single patient, we observed that colistin resistance in a single *E. coli* strain evolved through two unique variants in *basRS*.

We show that SDD can select for colistin resistance in species that are not intrinsically colistin-resistant. Continued surveillance for the emergence of colistin resistance in patients treated with SDD is needed to ensure the safety of this prophylactic therapy.

Introduction

Selective decontamination of the digestive tract (SDD) is a prophylactic antibiotic treatment used in Dutch intensive care units (ICUs) which lowers the mortality in ICU-admitted patients through the selective eradication of opportunistic pathogens in the oropharyngeal and gut microbiota. One of the targets of SDD are the Enterobacteriaceae, which are collectively responsible for a significant proportion of hospital-acquired infections (1–5). In SDD, a combination of the antibiotics colistin and tobramycin, and the antifungal amphotericin B, is applied to the digestive tract of ICU patients. In addition, during the first four days of ICU stay, patients are also intravenously administered a biliary-excreted third-generation cephalosporin, contributing to the eradication of Gram-negative pathogens from the gut (1). The use of SDD requires the surveillance for potential colonisation of the digestive tract of SDD-treated patients with tobramycin- and/or colistin-resistant Enterobacteriaceae (6).

Colistin is a cationic cyclic polypeptide with a fatty acid tail that specifically acts on Gram-negative bacteria. Colistin electrostatically interacts with the anionic phosphate groups of the lipid A moiety of lipopolysaccharide (LPS) molecules that comprise the majority of the outer leaflet of the outer membrane (7, 8). Through binding to the phosphate groups, and insertion of its hydrophobic domains, colistin destabilizes the outer membrane. After disruption of the outer membrane, colistin targets the LPS resident in the cytoplasmic membrane after its synthesis in the cytoplasm. The destabilization of the cytoplasmic membrane ultimately kills the cell (9–11).

The most frequent mechanisms of colistin resistance involve the reduction of the anionic charges of lipid A, which reduces the electrostatic interactions between colistin and LPS. This is achieved by the covalent linkage of positively charged groups like phosphoethanolamine or 4-amino-4-deoxy-L-arabinose to the phosphate groups of lipid A (11). Addition of these groups to lipid A is achieved by the products of the EptA and Arn operons, respectively (12). The transcriptional activity of these operons is controlled by the PhoPQ and PmrAB (BasRS in *Escherichia coli*) two-component regulatory systems. The permanent activation of these two-component regulatory systems may thus result in colistin resistance. Mutations that result in activation of these two-component regulatory systems often occur in specific hotspots (e.g. G53 and R81 in BasR/PmrA, and A159 in BasS/PmrB) (13, 14). Continued surveillance for colistin resistance has led to the discovery of novel mechanisms of colistin resistance, including L10 substitutions in BasS/PmrB (15), but most importantly the acquisition of *mcr* genes (16). For *E. coli*, the acquisition of *mcr*-carrying mobile genetic elements is a particularly

important mechanism through which colistin resistance may occur (17). Other mechanisms of acquired colistin resistance in Enterobacteriaceae include the production of capsular polysaccharides (18), and efflux pump activity (19).

The use of colistin in SDD is controversial (20), due to the increasing importance of colistin as a last-resort antibiotic for the treatment of infections caused by multidrug-resistant Gram-negative pathogens. In addition, SDD has been associated with outbreaks of Gram-negative bacteria with acquired colistin resistance in ICUs (21, 22). There is thus a particular interest in understanding the spread of colistin-resistant strains and to characterise the mechanisms that cause colistin resistance in Enterobacteriaceae from SDD-treated patients. In this work, we analyse thirteen colistin-resistant strains (nine *E. coli* strains, three *Klebsiella aerogenes* strains, and one *Enterobacter asburiae* strain) from SDD-treated ICU patients through whole genome sequencing, and investigate the mechanisms that have contributed to colistin resistance.

Results

Strains with acquired colistin resistance are rarely isolated during SDD.

As described in our previous work (23), 388 Gram-negative strains were isolated from 1105 rectal swabs, from 428 patients receiving SDD. Of these, 102 strains belonged to species that are intrinsically resistant to colistin. The remaining 286 isolates were suspected of having acquired colistin resistance because they survived during colistin-containing SDD treatment of the patients. These strains were initially tested for colistin susceptibility on Sensititre™ FRCOL plates (Thermo Fisher Scientific, Wesel, Germany). A total of ten *E. coli* strains, one *E. asburiae* strain, and three *K. aerogenes* strains were found to be resistant to colistin through this method. We then tested these strains for colistin susceptibility in a standardized broth microdilution assay and all strains were phenotypically resistant to colistin with the exception of *E. coli* strain 89, which was left out of further analyses (Table 1). Thus, we found thirteen strains (4.5% of the non-intrinsically resistant isolates) to be colistin-resistant. The colistin-resistant *K. aerogenes* and *E. coli* strains had minimal inhibitory concentration (MIC) values up to 32 µg/ml colistin.

Table 1: Characteristics and colistin MICs of strains used in this study.[a]

Strain	Date of isolation	Patient	Species	Colistin MIC (µg/ml)[b]
24	6 August 2018	37	*Klebsiella aerogenes*	0.25
25	6 August 2018	37	*Klebsiella aerogenes*	32
26	9 August 2018	37	*Klebsiella aerogenes*	32
27	9 August 2018	37	*Klebsiella aerogenes*	32
32	7 September 2018	307	*Enterobacter asburiae*	8192
89	26 July 2018	337	*Escherichia coli*	0.25
137	30 August 2018	27	*Escherichia coli*	0.125
138	30 August 2018	27	*Escherichia coli*	8
260	1 November 2018	31	*Escherichia coli*	16
262	1 November 2018	31	*Escherichia coli*	16
263	1 November 2018	311	*Escherichia coli*	4
274	5 November 2018	31	*Escherichia coli*	16
281	8 November 2018	31	*Escherichia coli*	16
292	14 November 2018	31	*Escherichia coli*	16
296	14 November 2018	31	*Escherichia coli*	16
297	14 November 2018	31	*Escherichia coli*	16

[a] Overview of the isolates used in this study, the date of isolation, the patient number, the species of the isolate as determined by MALDI-TOF on a Bruker microflex system (Leiderdorp, the Netherlands).
[b] The MIC value of the broth microdilution method displays the median value of three independent replicates in triplicate.

The MIC of the *E. asburiae* strain was found to reach values up to 8192 µg/ml colistin. For patients 27 and 37, colistin-susceptible strains of the same species were also isolated from rectal swabs during surveillance (Figure 1).

In total, five of the 428 patients (1.2%) tested positive for an isolate with acquired colistin resistance. Of the ten *E. coli* strains, seven were isolated from one patient, the remaining three strains originated from three other patients. The three *K. aerogenes* strains were isolated from a single patient. None of the patients carried multiple colistin-resistant species. Of the five patients from whom a strain with acquired colistin resistance was isolated, only patient 307 carried a colistin-resistant strain (*E. asburiae*) at the start of ICU admission, suggesting that this strain had acquired colistin resistance before the SDD treatment (Figure 1). This strain was no longer present on any of the following sampling time-points. Patients 311 and 27 initially tested negative for colistin-resistant strains, but tested positive for colistin-resistant strains at 2 and 3 days after ICU admission, respectively. Following sampling time-points were negative for colistin-resistant strains. Patient 37 was found to be colonised by colistin-resistant *K. aerogenes* at day 15, and day 18, of ICU hospitalisation. Subsequent screening from day 22 to discharge from the ICU on day 75, did not reveal the presence of strains that had acquired colistin resistance. Patient 31 was first found to be colonised by colistin-resistant *E. coli* at day 9 of ICU hospitalisation, and with exception of screening on day 20, remained colonised up to the moment the patient was lost to follow-up at day 24.

Colistin-resistant strains from ICU patients have a diverse genetic background and carry a variety of acquired antibiotic-resistance genes

We assessed whether a single or multiple clones of colistin-resistant *E. coli* and *K. aerogenes* strains were colonising the ICU patients, by constructing a phylogenetic tree based on the core genome of each of the species, using

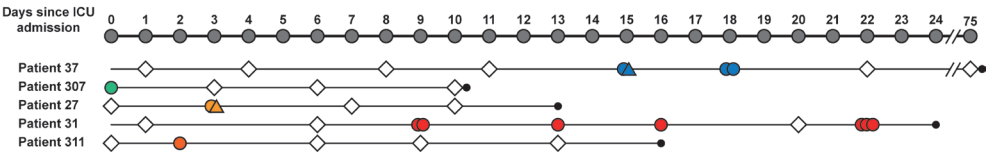

Figure 1: Timeline of rectal swabs collected from SDD-treated ICU patients and isolation of colistin-susceptible, or colistin-resistant strains. Filled circle: isolation of colistin-resistant strain from rectal swab. Filled triangle; a colistin-susceptible strain of the same species as the colistin-resistant strain was isolated from the rectal swab. Open diamonds, no naturally colistin-susceptible Gram-negative bacteria were isolated. The symbols are colour coded according to the species of the isolated strain: *Klebsiella aerogenes*, blue; *Enterobacter asburiae*, green; *Escherichia coli*, yellow, orange and red. Multiple symbols on the same day indicate the isolation of multiple strains from the same swab. The length of ICU admission is indicated by a line, and discharge is indicated by a circle at the end of the line.

the genomes sequenced in this study and by a collection of publicly available genomes. Based on the core-genome phylogeny for *E. coli*, we observed that three distinct clones of colistin-resistant *E. coli* colonised the three individual patients (Figure 2A). The colistin-susceptible *E. coli* strain 137 from patient 27 did not cluster with colistin-resistant *E. coli* strain 138 isolated from the same patient. All *K. aerogenes* strains, isolated from patient 37, belonged to a single clone (Figure 2B).

We screened the assembled genomes of the colistin-resistant strains for acquired antibiotic resistance genes (Figure 2C). We identified that only strain *E. coli* strain 138 was positive for the *mcr-1.1* gene. The *mcr-1.1* gene was located as the sole antibiotic resistance gene on a 59.5 kbp contig, containing an IncI2-type replicon. The sequence of this contig shared 99% similarity with plasmid sequences obtained from multiple courses, including from a *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain from a patient from China (accession number MH522416.1) (24), and an *E. coli* strain isolated from chicken faeces in Thailand (accession number MG557851.1) (25), illustrating the global spread of this plasmid. In addition, we found that the colistin-resistant *E. coli* strains carried between 2 and 12 antibiotic resistance genes, including to aminoglycosides and β-lactams. The *K. aerogenes* strains only carried the fosfomycin resistance gene *fosA*, and the single *E. asburiae* strain had *fosA* and the AmpC-type β-lactamase *blaACT-4*.

In-patient microevolution of colistin resistance in *Escherichia coli* during SDD.

To investigate the possible microevolutionary events in colistin resistance in Enterobacteriaceae during gut colonisation, we investigated the genetic diversity between the four clonally related *K. aerogenes* strains from patient 37, and the seven colistin-resistant *E. coli* strains isolated from patient 31. By comparing the genome of the single colistin-susceptible *K. aerogenes* strain with the genomes of the colistin-resistant strains, we determined that the colistin-resistant strains 25, 26, and 27 had a G53S substitution in the PmrA transcriptional regulator of the PmrAB two-component regulatory system. This substitution has previously been described to cause colistin resistance in *K. aerogenes* (26). In addition, we observed an insertion of a single guanine nucleotide in the gene encoding the sulphate adenylyltransferase subunit 2 CysD, involved in sulphate assimilation. CysD has not been previously described in relation to colistin resistance. No mutations differentiating the three colistin-resistant strains were observed.

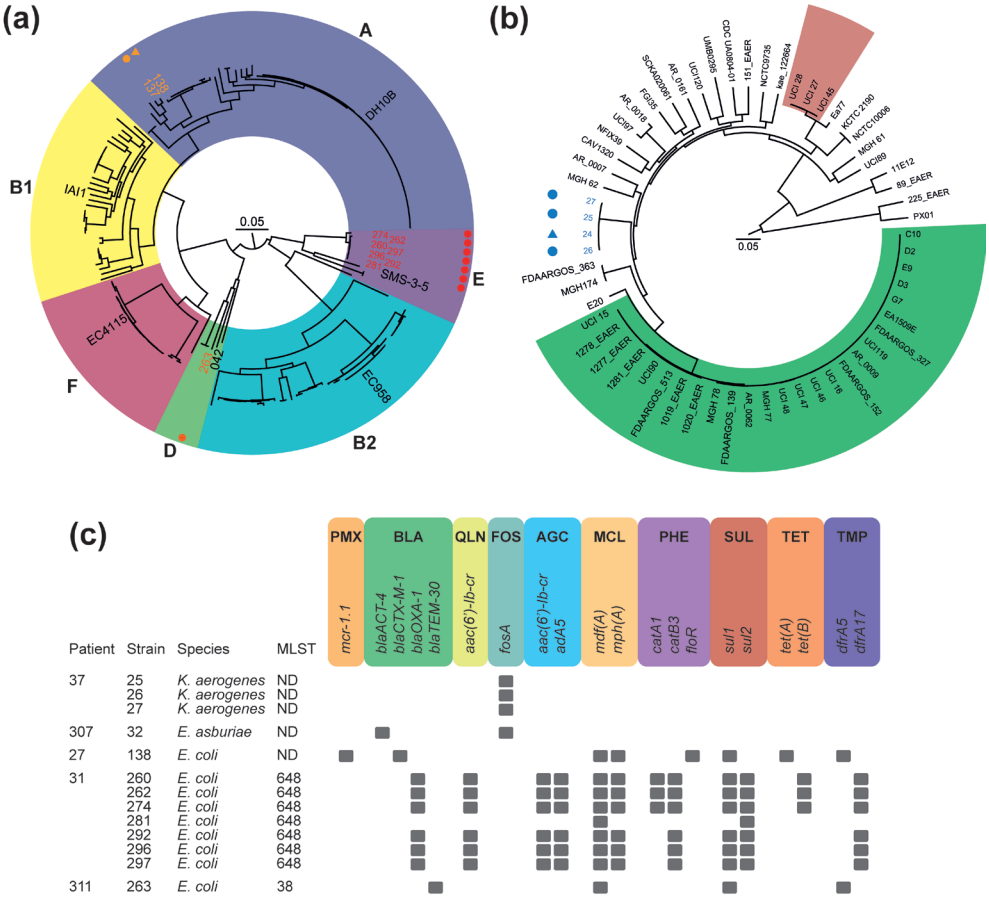

Figure 2: Phylogenetic inferences and acquired resistance genes of colistin-resistant *E. coli* and *K. aerogenes*. A) The phylogenetic tree of *E. coli* represents the core genome alignment (2.1 Mbp) of 198 genomes (178 genomes from public databases, the genomes of the nine colistin-resistant strains, and the two colistin-susceptible strains). One representative reference strain per *E. coli* phylogroup is indicated (67). Phylogroup A is coloured dark blue; B1, yellow; B2, light blue; D, green; E, purple; F, pink. Colistin-susceptible and colistin-resistant strains are indicated by filled triangles and circles respectively. The studied strains are highlighted with colours corresponding to Figure 1. **B)** The phylogenetic tree of *K. aerogenes* represents a core genome alignment (1.6 Mbp) of 56 publicly available genomes, and the three colistin-resistant strains described in this study. Colistin-susceptible and resistant strains are indicated by filled triangles and circles respectively. The studied strains are highlighted with colours corresponding to Figure 1. The genomes of the dominant *K. aerogenes* ST4 and ST93 lineages associated with infections have an red and green background respectively (68). **C)** Acquired antibiotic resistance genes of colistin-resistant strains. Strains are grouped according to the patient from which they were isolated. Species and MLST type are indicated per strain. Antibiotic resistance genes in the genomes of the colistin-resistant strains were detected by ResFinder 3.2 (60). Classes of antibiotic resistance genes are abbreviated as follow: PMX, polymyxin resistance; BLA, ß-lactam resistance; QLN, quinolone resistance; FOS, fosfomycin resistance; AGC, aminoglycoside resistance; MCL, macrolide, lincosamide, and streptogramin B resistance; PHE, phenicol resistance; SUL, sulfonamide resistance; TET, tetracycline resistance; TMP, trimethoprim resistance. ND; not determined.

The seven clonally related colistin-resistant *E. coli* isolates from patient 31 were obtained on four separate days, over a two-week period (Table 1, Figure 2A). Within these strains, we observed that the strains isolated on 1 and 5 November 2018 (strains 260, 262, and 274) had a G53A substitution in the BasR transcriptional regulator of the BasRS two-component regulatory system (Figure 3A). This substitution has previously been experimentally proven to contribute to colistin resistance in *E. coli* (14). The strains isolated after November 5, 2018 (strains 281, 292, 296, and 297) however, did not encode the G53A BasR substitution. Instead, we observed an 18 bp deletion (nucleotides 10 through 27) in *basS*, leading to the deletion of six amino acids (4 through 9) at the N-terminal end of BasS. A limited number of additional mutations within these isolates were found. Two additional mutations differentiated the strains with the G53A BasR substitution. Strain 260 had a Q260X substitution in the phosphoanhydride phosphohydrolase AppA, and strain 262 had a N179S substitution in formate hydrogenlyase subunit 5 HycE. These two proteins have not been described in relation to colistin resistance.

The three variants of this strain from November 14th (strains 292, 296, and 297) had a mucoid phenotype (Figure 3B). Between the genome of the non-mucoid strain carrying the 18 bp deletion (strain 281), and the genomes of the mucoid strains, we identified an I424S substitution in YrfF (an homolog of *Salmonella* IgaA). YrfF is a negative regulator of the Rcs phosphorelay system, and is thus involved in regulation of capsule production (27).

To establish the impact of colistin resistance on fitness, we determined the maximum specific growth rate of the different strains. In the strains isolated from patient 31, we observed a reduction of about 15% in maximum specific growth rate from the strains that harbor the G53A BasR substitution compared to those harboring the 18 bp deletion in *basS* (Figure 3C). An similar decrease in maximum specific growth rate is observed in the strains that have the I424S substitution in YrfF. For the *K. aerogenes* strains isolated from patient 31 that had the G53S PmrA substitution, we did not observe a change in the maximum specific growth rate (Supplemental Figure S1).

Mutations in *basS* can lead to reduced susceptibility to colistin

As described above, for the strains isolated from patients 27, 31, and 37, we could determine the probable variants that led to colistin resistance. For *E. coli* strain 263, the absence of an isogenic, colistin-susceptible strain made this difficult. We used Enterobase to determine that *E. coli* strain eo2071 (barcode ESC_BA7113AA) is most closely related to strain 263. We then compared the sequence of *basRS*

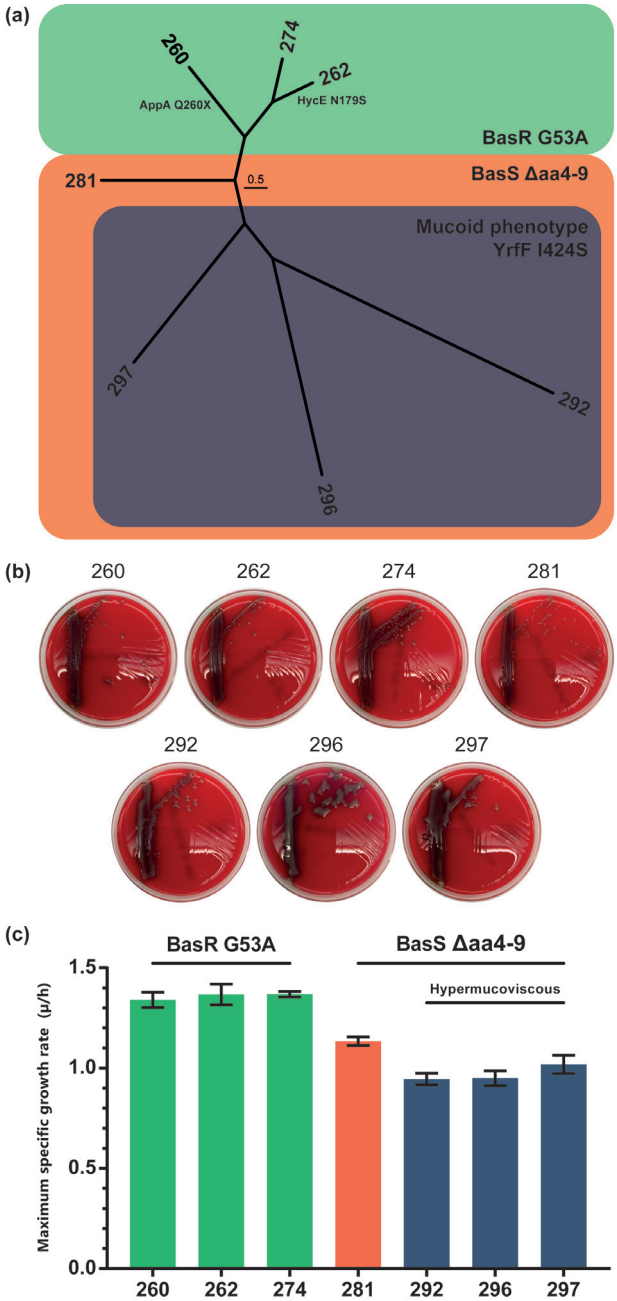

Figure 3: *In vivo* microevolution of colistin-resistant *E. coli* strains isolated from patient 31. A) Cladogram of phylogenetic relations between the strains isolated from patient 31. Branches with the BasR G53A substitution, the BasS Δaa4-9 deletion, and the I424S substitution in YrfF are indicated through a green, orange, and purple background respectively. Other mutations between strains are indicated next to the applicable branch. **B)** Growth phenotypes of the *E. coli* strains isolated from patient 31 on TSA plates with 5% sheep blood after overnight growth at 37°C. **C)** Maximum specific growth rate of colistin-resistant *E. coli* strains isolated from patient 31. The values presented represent mean with standard deviation, of three independent experiments, performed in duplicate.

of the colistin-resistant *E. coli* strain 263 with *basRS* of eo2071, leading to the identification of a G19E substitution in BasS in strain 263.

We aimed to investigate the relevance of the 18 bp deletion in *basS* and the mutation leading to the G19E substitution for colistin resistance in *E. coli*. Due to the multidrug-resistant nature of the nosocomial colistin-resistant strains, we constructed chromosomal integration mutants of the genes encoding the mutated BasRS two-component regulatory system in the *att*Tn*7* site in the BW25113 derived Δ*basRS* strain BW27848. We constructed sequences that consisted of the promotor region of the *eptA-basR-basS* operon and the *basRS* coding sequences of the nosocomial colistin-resistant strain to complement BW27848 using the Tn*7* transposon system, as described previously (14). MIC determinations of the chromosomal integration mutants show that introduction of the *basRS* alleles of the nosocomial colistin-resistant strains led to reduced susceptibility to colistin (Table 2). The MIC value of the BW27848 strain with the insertion of *basRS* encoding the 18 bp deletion in *basS* increased 4-fold. The MIC value of the BW27848 strain with the *basRS* encoding the G19E substitution in *basR* increased 2-fold. Restoring the deleted 18 basepairs to *basS*, or reversing the G19E BasR substitution, returned the colistin susceptibility to BW25113 levels.

Table 2: Mutations in *basS* and *basR* result in reduced susceptibility to colistin.[a]

Strain	Colistin MIC (µg/ml)[b]
BW25113	0.125
BW27848	0.25
BW25113::Tn*7* Empty	0.125
BW27848::Tn*7* Empty	0.125
BW27848::Tn*7* BW25113	0.125
BW27848::Tn*7* 281	1
BW27848::Tn7 281 BasS Restored	0.125
BW27848::Tn*7* 263	0.25
BW27848::Tn7 263 BasR E19G	0.125

[a] Transgene insertion mutants, encoding the *basRS* alleles found in the colistin-resistant strains in this study, and mutated alleles in which these mutations were reversed, were inserted into the *att*Tn*7* site of *E. coli* BW27848 (the Δ*basRS* mutant of BW25113). The strain name (in italics) indicates that the construct has been modified through inverse PCR site directed mutagenesis to reverse the mutation associated with colistin resistance.
[b] The values presented represent the median of three independent experiments, performed in triplicate.

Discussion

In this study, we show that the carriage rate of strains with acquired colistin resistance among ICU patients (1.2%) was similar to rates found previously in the Netherlands, in- or outside ICU settings (5, 14, 28). In total, thirteen strains, from three species, with acquired colistin resistance were isolated (4.5% of non-intrinsically resistant isolates). We found multiple distinct clones of colistin-resistant strains among patients, but within a patient strains were clonally related. One clone belonged to the emerging ESBL-carrying, multidrug-resistant ST648 lineage (29). The development of colistin resistance in these *E. coli* strains can be explained by the acquisition of *mcr-1.1*, a G53A or G19E substitution in BasR, or an 18 bp deletion in *basS*. The 18 bp deletion in *basS* was associated with a loss of fitness. Development of colistin resistance in the *K. aerogenes* strains was associated with a G53S substitution in PmrA. The mechanism of colistin resistance in the *E. asburiae* strain was not identified.

Through the longitudinal sampling, selection and microevolutionary processes related to prolonged exposure to colistin could be examined. We found that colistin resistance in a clonal *E. coli* population can emerge through two different mutational trajectories in *basRS*, as observed in the strains obtained from patient 31. The observation of distinct variants in *basRS* contributing to colistin resistance in a clonal *E. coli* population, reflects the ability of *E. coli* to quickly adapt to novel niches and selective pressures through multiple evolutionary pathways (30–32). Subsequent prolonged colonisation will result in genetic drift and occurrence of mutations that are presumably neutral to colistin resistance, as we observed with mutations in AppA and HycE (33). We propose that this *E. coli* clone was able to persistently colonise the patient because it carried mechanisms that confer resistance to the antibiotics (cephalosporins, tobramycin, and colistin) used in SDD.

We observe transient colonisation by an *mcr-1.1* carrying *E. coli* in a patient receiving colistin-containing treatment. Transient colonisation of the gut by *mcr*-carrying *E. coli* has previously been observed, in the context of international travel, and may reflect the decreased fitness of *E. coli* strains carrying *mcr* genes (34–36). We also observed high-level colistin resistance in an *Enterobacter asburiae* strain, a member of the *E. cloacae* complex (37), and a clonal population of colistin-resistant *K. aerogenes*. A limited number of high-level resistant *E. asburiae* (38–42), and *K. aerogenes* (26, 43–45) strains have been described before. However, the exact mechanisms through which colistin resistance may evolve in these species remains poorly studied (26, 46).

Prolonged exposure to a selective pressure (e.g. antibiotics) will select for resistance, which generally comes at a fitness cost. Fitness of an antibiotic-resistant clone can subsequently increase due to the accumulation of compensatory mutations (47). However, we observed a reduction of the maximum specific growth rate in the *E. coli* strains in which *yrfF* was mutated. We hypothesise that this mutation is likely to be responsible for the mucoid phenotype in these strains, through increased biosynthesis of capsular polysaccharides, which will negative impact maximum specific growth rate due to the fitness cost of capsule biosynthesis. While mucoidy has been linked to colistin resistance in *K. pneumoniae*, and *Neisseria meningitides* (18, 48, 49), we did not observe a difference in the colistin MICs of clonally related non-mucoid and mucoid *E. coli* strains. Mucoidy in *E. coli* is a relatively poorly understood phenotype. However, the *in vitro* determination of the maximum specific growth rate as a marker for fitness could inaccurately reflect the effects of this mutation *in vivo*, where host-pathogen interactions and limitations in substrates, might give a fitness advantage to the strain that harbours the 18 bp deletion in *basS*, and additional advantages to strains with the observed mucoid phenotype, including increased survival under influence of humoral and cellular immune responses (50, 51).

In this study, we find that Gram-negative opportunistic pathogens carried in the gut of patients can acquire colistin resistance, either through mutation of genes that regulate lipid A modifications or by the acquisition of the *mcr-1.1* gene. However, the low prevalence of colistin-resistant strains in ICU-administered patients suggests that the evolution of colistin resistance is currently of minor concern during the implementation of SDD in Dutch ICUs. As the prevalence of multidrug-resistant Gram-negative bacteria in the Netherlands are low, strains colonising patients will be generally susceptible to one or more of the antibiotics used in SDD. Indeed, in four patients, strains with acquired colistin resistance are rapidly eradicated from the gut. However, the long-term colonisation of patient 31 with an *E. coli* clone that is colistin-resistant and carries genes conferring resistance to the other antibiotics used in SDD, indicates that SDD can select for multidrug-resistant Gram-negative bacteria in the gut of ICU patients. The risk of the emergence of colistin resistance in the patient gut may be more pronounced in countries where higher rates of circulating antibiotic resistant bacteria are observed, or in settings with failing infection control (5, 21, 52, 53). Routine surveillance is thus vital to thwart selection and spread of multidrug-resistant strains upon SDD. Further studies are required to better understand the diversity of mechanisms of acquired colistin resistance in clinical Enterobacteriaceae isolates, particularly in species like *E. asburiae*, and *K. aerogenes* for which the colistin resistance mechanisms have so far been poorly studied.

Material and methods

Bacterial strains, growth conditions, chemicals, plasmid isolation, and oligonucleotide primers

Colistin-resistant strains were isolated from patients receiving SDD that were admitted to the intensive care unit of the University Medical Centre Utrecht, Utrecht, the Netherlands, between July 2018 to January 2019, as previously described (23). We included colistin-susceptible strains of the same species that were isolated from the same patient at the same day from which a colistin-resistant strain was isolated. *E. coli* strain BW25113 and the BW25113-derived Δ*basRS* strain BW27848 from the Keio collection were obtained from the Coli Genetic Stock Center (54, 55). All strains were grown in Lysogeny Broth (LB; Oxoid, Landsmeer, the Netherlands) at 37°C with agitation at 300 rpm unless otherwise noted. Strains containing pGRG36 were grown at 30°C (56). When appropriate, kanamycin (50 µg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands), and ampicillin (100 µg/ml; Sigma-Aldrich) were used. Colistin sulphate was obtained from Duchefa Biochemie (Haarlem, the Netherlands). The L-(+)-arabinose was obtained from Sigma-Aldrich. Plasmids were purified using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, Landsmeer, the Netherlands). Oligonucleotide primers (Supplemental Table S1) were obtained from Integrated DNA Technologies (Leuven, Belgium).

Determination of minimal inhibitory concentration

MICs of colistin were determined using a broth microdilution method in line with EUCAST guidelines (57), as previously described (14, 58). The breakpoint values for colistin resistance (MIC > 2 µg/ml) in Enterobacteriaceae were obtained from the 2019 European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST; http://www.eucast.org/clinical_breakpoints/).

Genomic DNA isolation and whole-genome sequencing

Genomic DNA was isolated, and checked for quality, as described previously.(14) Sequence libraries for Illumina sequencing were prepared using the Nextera XT kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Libraries were sequenced on an Illumina NextSeq 500 system with a 300-cycle (2 × 150 bp) NextSeq 500/550 Mid Output v2.5 kit.

Genome assembly, MLST typing, and identification of antibiotic resistance genes

Illumina sequencing data were assessed (FastQC v0.11.7), and trimmed (nesoni v0.115) for quality, and used for *de novo* genome assembly (SPAdes v3.12.0), as described before (14, 59). MLST typing was performed using the mlst package v2.10 (https://github.com/tseemann/mlst). Assembled contigs were screened for acquired antibiotic resistance genes using ResFinder 3.2 using standard settings (60).

Construction of core genome phylogenetic trees.

Genome assemblies of the sequenced strains were aligned with publicly available genomes of the same species obtained from NCBI databases (Supplemental Table S2). Conserved regions of genomes were identified and aligned using Parsnp v1.2 (61). FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the phylogenetic tree.

Determination of SNPs and indels through short-read sequences

Read-mapping of Nesoni-filtered reads was performed using Bowtie2 (62). Single nucleotide polymorphism (SNP) and insertion and deletion (indel)-calling was performed using SAMtools 0.1.19 (63) through the settings described before (64). Identified SNPs and indels were manually linked to features in the Prokka annotation, and inspected for synonymous versus non-synonymous mutations. Identified mutations SNPs and indels were confirmed by performing PCR (oligonucleotide primers listed in Supplemental Table S1), and Sanger sequencing (Macrogen, Amsterdam, the Netherlands)

Genome comparisons through Enterobase

To identify mutations that could potentially contribute to colistin resistance in the *E. coli* strains 260 and 263, Raw Illumina sequence reads were uploaded to, and assembled by, Enterobase (65) under barcode ESC_OA6301AA and ESC_OA6302AA respectively. The *E. coli* cgMLST scheme was used to define a closest relative in Enterobase and the assemblies of these strains were then used to compare the genome sequences of the colistin-resistant strains described here and the closest relative in Enterobase. Because of the clonal relatedness of the

strains isolated from patient 31, only one strain was submitted to Enterobase for this analysis. Identified sequence variations in the colistin-resistant strains were confirmed by performing PCR (oligonucleotide primers listed in Supplemental Table S1), and Sanger sequencing (Macrogen, Amsterdam, the Netherlands)

Construction of chromosomal *basRS* transgene insertions

Single-copy chromosomal transgene insertion mutants of *basRS*, derived from clinical strains, were constructed in BW27848 using the Tn*7* transposon system located on the pGRG36 plasmid (56, 66), as previously described (14).

Determination of maximum specific growth rate

To determine the maximum specific growth rate, a Bioscreen C instrument (Oy Growth Curves AB, Helsinki, Finland) was used. Overnight cultures were used to inoculate 200 µl fresh LB medium 1:1000. Incubation was set at 37°C with continuous shaking set to have maximum amplitude and fastest speed. Growth was observed by measuring the absorbance at 600 nm every 10 minutes.

Data availability

Sequence data has been deposited in the European Nucleotide Archive (accession number PRJEB34028).

Statistical analysis

Where applicable, statistical significance was determined using the parametric one-way ANOVA test. Correction for multiple comparison testing was performed for with a Tukey correction. Family-wise significance was defined as a p-value < 0.05.

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Transparency

The authors disclose no conflicts of interest.

Author contributions

A.B.J. conceived and designed experiments, performed experiments, analyzed data, and wrote the manuscript. D.v.H. reviewed patients records and microbiological data. M.J.M.B. wrote the manuscript. R.J.L.W. wrote the manuscript. W.v.S. conceived and designed experiments, wrote the manuscript, and supervised the study. All authors reviewed and approved the final version of the manuscript.

References

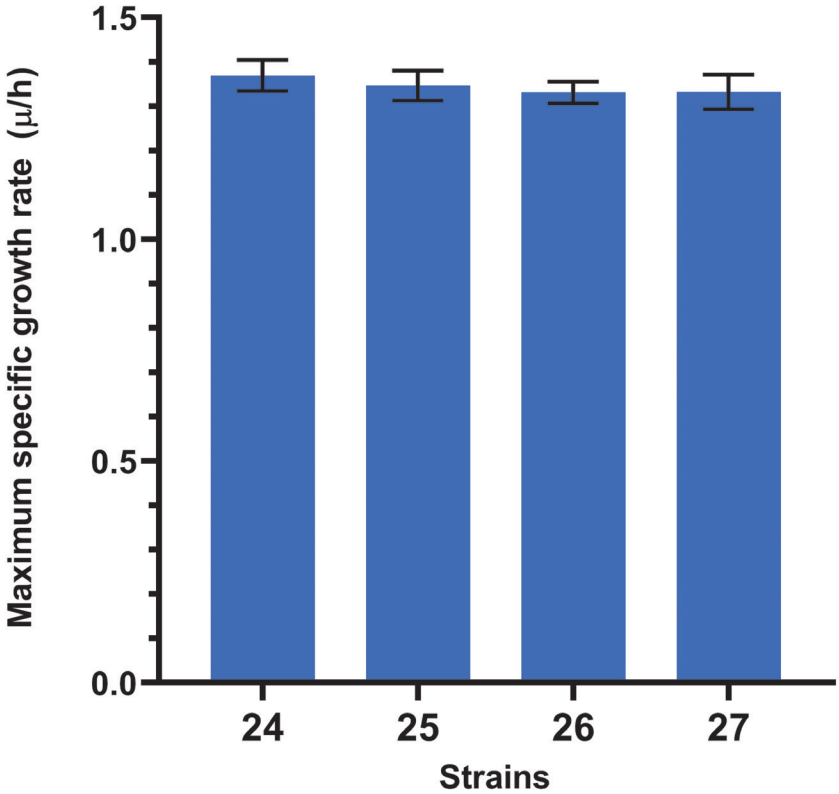
1. de Smet AMGA, Kluytmans JAJW, Cooper BS, Mascini EM, Benus RFJ, van der Werf TS, van der Hoeven JG, Pickkers P, Bogaers-Hofman D, van der Meer NJM, Bernards AT, Kuijper EJ, Joore JCA, Leverstein-van Hall MA, Bindels AJGH, Jansz AR, Wesselink RMJ, de Jongh BM, Dennesen PJW, van Asselt GJ, te Velde LF, Frenay IHME, Kaasjager K, Bosch FH, van Iterson M, Thijsen SFT, Kluge GH, Pauw W, de Vries JW, Kaan JA, Arends JP, Aarts LPHJ, Sturm PDJ, Harinck HIJ, Voss A, Uijtendaal E V, Blok HEM, Thieme Groen ES, Pouw ME, Kalkman CJ, Bonten MJM. 2009. Decontamination of the digestive tract and oropharynx in ICU patients. N Engl J Med 360:20–31.
2. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections summary of data reported to the national healthcare safety network at the Centers for Disease Control and Prevention, 2009–2010. Infect Control Hosp Epidemiol 34:1–14.
3. Peleg AY, Hooper DC. 2011. Hospital-acquired infections due to Gram-negative bacteria. N Engl J Med 362:1804–1813.
4. Wittekamp BH, Plantinga NL, Cooper BS, Lopez-Contreras J, Coll P, Mancebo J, Wise MP, Morgan MPG, Depuydt P, Boelens J, Dugernier T, Verbelen V, Jorens PG, Verbrugghe W, Malhotra-Kumar S, Damas P, Meex C, Leleu K, Van Den Abeele AM, Gomes Pimenta De Matos AF, Fernández Méndez S, Vergara Gomez A, Tomic V, Sifrer F, Villarreal Tello E, Ruiz Ramos J, Aragao I, Santos C, Sperning RHM, Coppadoro P, Nardi G, Brun-Buisson C, Bonten MJM. 2018. Decontamination strategies and bloodstream infections with antibiotic-resistant microorganisms in ventilated patients: a randomized clinical trial. J Am Med Assoc 320:2087–2098.
5. Oostdijk EAN, Kesecioglu J, Schultz MJ, Visser CE, De Jonge E, Van Essen EHR, Bernards AT, Purmer I, Brimicombe R, Bergmans D, Van Tiel F, Bosch FH, Mascini E, Van Griethuysen A, Bindels A, Jansz A, Van Steveninck FAL, Van Der Zwet WC, Fijen JW, Thijsen S, De Jong R, Oudbier J, Raben A, Van Der Vorm E, Koeman M, Rothbarth P, Rijkeboer A, Gruteke P, Hart-Sweet H, Peerbooms P, Winsser LJ, Van Elsacker-Niele AMW, Demmendaal K, Brandenburg A, De Smet AMGA, Bonten MJM. 2014. Effects of decontamination of the oropharynx and intestinal tract on antibiotic resistance in ICUs a randomized clinical trial. J Am Med Assoc 312:1429–1437.
6. Wittekamp BHJ, Oostdijk EAN, Cuthbertson BH, Brun-Buisson C, Bonten MJM. 2019. Selective decontamination of the digestive tract (SDD) in critically ill patients: a narrative review. Intensive Care Med.
7. Velkov T, Thompson PE, Nation RL, Li J. 2010. Structure-activity relationships of polymyxin antibiotics. J Med Chem 53:1898–1916.
8. Domingues MM, Inácio RG, Raimundo JM, Martins M, Castanho MARB, Santos NC. 2012. Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. Biopolymers 98:338–344.
9. Putker F, Bos MP, Tommassen J. 2015. Transport of lipopolysaccharide to the Gram-negative bacterial cell surface. FEMS Microbiol Rev 39:985–1002.
10. Sabnis A, Klöckner A, Becce M, Evans LE, Furniss RCD, Mavridou DAI, Stevens MM, Edwards AM. 2018. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. bioRxiv 479618v2.
11. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. 2011. Molecular mechanisms of antibiotic resistance. Chem Commun 47:4055–4061.
12. Simpson BW, Trent MS. 2019. Pushing the envelope: LPS modifications and their consequences. Nat Rev Microbiol 17:403–416.
13. Bourrel AS, Poirel L, Royer G, Vuillemin X, Kieffer N, Clermont O, Denamur E, Nordmann P, Decousser J. 2019. Colistin resistance in Parisian inpatient faecal *Escherichia coli* as the result of two distinct evolutionary pathways. J Antimicrob Chemother 74:1521–1530.
14. Janssen AB, Bartholomew TL, Marciszewska NP, Bonten MJM, Willems RJL, Bengoechea JA, van Schaik W. 2020. Nonclonal emergence of colistin resistance associated with mutations in the BasRS two-component system in *Escherichia coli* bloodstream isolates. mSphere 5:e00143-20
15. Cannatelli A, Giani T, Aiezza N, Pilato V Di, Principe L, Luzzaro F, Galeotti CL, Rossolini GM. 2017. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. Sci Rep 7:5071.
16. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin

resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168.
17. Wang R, van Dorp L, Shaw LP, Bradley P, Wang Q, Wang X, Jin L, Zhang Q, Liu Y, Rieux A, Dorai-Schneiders T, Weinert LA, Iqbal Z, Didelot X, Wang H, Balloux F. 2018. The global distribution and spread of the mobilized colistin resistance gene *mcr-1*. Nat Commun 9:1179.
18. Llobet E, Tomás JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154:3877–3886.
19. Padilla E, Llobet E, Doménech-Sánchez A, Martínez-Martínez L, Bengoechea JA, Albertí S. 2010. Klebsiella pneumoniae AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob Agents Chemother 54:177–183.
20. Bastin AJ, Ryanna KB. 2009. Use of selective decontamination of the digestive tract in United Kingdom intensive care units. Anaesthesia 64:46–49.
21. Halaby T, Al Naiemi N, Kluytmans J, van der Palen J, Vandenbroucke-Grauls CMJE. 2013. Emergence of colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. Antimicrob Agents Chemother 57:3224–3229.
22. Lübbert C, Faucheux S, Becker-Rux D, Laudi S, Dürrbeck A, Busch T, Gastmeier P, Eckmanns T, Rodloff AC, Kaisers UX. 2013. Rapid emergence of secondary resistance to gentamicin and colistin following selective digestive decontamination in patients with KPC-2-producing *Klebsiella pneumoniae*: a single-centre experience. Int J Antimicrob Agents 42:565–570.
23. van Hout D, Janssen AB, Rentenaar RJ, Vlooswijk JPM, Boel CHE, Bonten MJM. 2019. The added value of the selective SuperPolymyxin™ medium in detecting rectal carriage of Gram-negative bacteria with acquired colistin resistance in intensive care unit patients receiving selective digestive decontamination. Eur J Clin Microbiol Infect Dis 39:265-271
24. Lu X, Zeng M, Xu J, Zhou H, Gu B, Li Z, Jin H, Wang X, Zhang W, Hu Y, Xiao W, Zhu B, Xu X, Kan B. 2019. Epidemiologic and genomic insights on *mcr-1*-harbouring *Salmonella* from diarrhoeal outpatients in Shanghai, China, 2006–2016. EBioMedicine 42:133–144.
25. Yang Q, Li M, Spiller OB, Andrey DO, Hinchliffe P, Li H, MacLean C, Niumsup P, Powell L, Pritchard M, Papkou A, Shen Y, Portal E, Sands K, Spencer J, Tansawai U, Thomas D, Wang S, Wang Y, Shen J, Walsh T. 2017. Balancing *mcr-1* expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms. Nat Commun 8:2054.
26. Diene SM, Merhej V, Henry M, El Filali A, Roux V, Robert C, Azza S, Gavory F, Barbe V, La Scola B, Raoult D, Rolain JM. 2013. The rhizome of the multidrug-resistant *Enterobacter aerogenes* genome reveals how new "killer bugs" are created because of a sympatric lifestyle. Mol Biol Evol 30:369–383.
27. Majdalani N, Gottesman S. 2005. The Rcs phosphorelay: a complex signal transduction system. Annu Rev Microbiol 59:379–405.
28. De Jonge E, Schultz MJ, Spanjaard L, Nesbitt I. 2004. Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. CPD Anaesth 6:41–43.
29. Schaufler K, Semmler T, Wieler LH, Trott DJ, Pitout J, Peirano G, Bonnedahl J, Dolejska M, Literak I, Fuchs S, Ahmed N, Grobbel M, Torres C, McNally A, Pickard D, Ewers C, Croucher NJ, Corander J, Guenther S. 2019. Genomic and functional analysis of emerging virulent and multidrug-resistant *Escherichia coli* lineage sequence type 648. Antimicrob Agents Chemother 63:e00243-19.
30. Barroso-Batista J, Sousa A, Lourenço M, Bergman ML, Sobral D, Demengeot J, Xavier KB, Gordo I. 2014. The first steps of adaptation of *Escherichia coli* to the gut are dominated by soft sweeps. PLoS Genet 10:e1004182.
31. Lescat M, Launay A, Ghalayini M, Magnan M, Glodt J, Pintard C, Dion S, Denamur E, Tenaillon O. 2017. Using long-term experimental evolution to uncover the patterns and determinants of molecular evolution of an *Escherichia coli* natural isolate in the streptomycin-treated mouse gut. Mol Ecol 26:1802–1817.
32. Giraud A, Arous S, De Paepe M, Gaboriau-Routhiau V, Bambou JC, Rakotobe S, Lindner AB, Taddei F, Cerf-Bensussan N. 2008. Dissecting the genetic components of adaptation of *Escherichia coli* to the mouse gut. PLoS Genet 4:e2.
33. Ghalayini M, Launay A, Bridier-Nahmias A, Clermont O, Denamur E, Lescat M, Tenaillona O. 2018. Evolution of a dominant natural isolate of *Escherichia coli* in the human gut over the course of a year suggests a neutral evolution with reduced effective population size. Appl Environ Microbiol 84:e02377-17.

34. Kantele A, Kuenzli E, Dunn S, Dance D, Newton P, Davong V, Mero S, Pakkanen S, Neumayr A, Hatz C, Snaith A, Kallonen T, Corander J, McNally A. 2019. Real-time sampling of travelers shows intestinal colonization by multidrug-resistant bacteria to be a dynamic process with multiple transient acquisitions. bioRxiv 827915.
35. Vasquez AM, Montero N, Laughlin M, Dancy E, Melmed R, Sosa L, Watkins LF, Folster JP, Strockbine N, Moulton-Meissner H, Ansari U, Cartter ML, Walters MS. 2016. Investigation of *Escherichia coli* harboring the *mcr-1* resistance gene — Connecticut, 2016. Morb Mortal Wkly Rep 65:979–980.
36. Tietgen M, Semmler T, Riedel-Christ S, Kempf VAJ, Molinaro A, Ewers C, Göttig S. 2018. Impact of the colistin resistance gene *mcr-1* on bacterial fitness. Int J Antimicrob Agents 51:554–561.
37. Brenner DJ, McWhorter AC, Kai A, Steigerwalt AG, Farmer JJ. 1986. *Enterobacter asburiae* sp. nov., a new species found in clinical specimens, and reassignment of *Erwinia dissolvens* and *Erwinia nimipressuralis* to the genus *Enterobacter* as *Enterobacter dissolvens* comb. nov., and *Enterobacter nimipressuralis* comb. nov.. J Clin Microbiol 23:1114–1120.
38. Pena I, Picazo JJ, Rodríguez-Avial C, Rodríguez-Avial I. 2014. Carbapenemase-producing Enterobacteriaceae in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among VIM-1-producing *Klebsiella pneumoniae* ST11 isolates. Int J Antimicrob Agents 43:460–464.
39. Rossi F, Girardello R, Cury AP, Di Gioia TSR, Almeida JN de, Duarte AJ da S. 2017. Emergence of colistin resistance in the largest university hospital complex of São Paulo, Brazil, over five years. Brazilian J Infect Dis 21:98–101.
40. Bradford PA, Kazmierczak KM, Biedenbach DJ, Wise MG, Hackel M, Sahm DF. 2016. Correlation of β-lactamase production and colistin resistance among Enterobacteriaceae isolates from a global surveillance program. Antimicrob Agents Chemother 60:1385–1392.
41. Boo TW, O'Connell N, Power L, O'Connor M, King J, McGrath E, Hill R, Hopkins KL, Woodford N. 2013. First report of IMI-1-producing colistin-resistant *Enterobacter* clinical isolate in Ireland, March 2013. Eurosurveillance 18:pii=20548.
42. Olaitan AO, Morand S, Rolain JM. 2016. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. Int J Antimicrob Agents 47:1–3.
43. Bollet C, Raoult D, Page J. 2005. Successive emergence of *Enterobacter aerogenes* strains resistant to imipenem and colistin in a patient. Antimicrob Agents Chemother 49:1354–1358.
44. Bedenić B, Vranić-Ladavac M, Venditti C, Tambić-Andrašević A, Barišić N, Gužvinec M, Karčić N, Petrosillo N, Ladavac R, di Caro A. 2018. Emergence of colistin resistance in *Enterobacter aerogenes* from Croatia. J Chemother 30:120–123.
45. Landman D, Salamera J, Quale J. 2013. Irreproducible and uninterpretable polymyxin B MICs for *Enterobacter cloacae* and *Enterobacter aerogenes*. J Clin Microbiol 51:4106–4111.
46. Telke AA, Olaitan AO, Morand S, Rolain JM. 2017. *soxRS* induces colistin hetero-resistance in *Enterobacter asburiae* and *Enterobacter cloacae* by regulating the *acrAB-tolC* efflux pump. J Antimicrob Chemother 72:2715–2721.
47. MacLean RC, Vogwill T. 2015. Limits to compensatory adaptation and the persistence of antibiotic resistance in pathogenic bacteria. Evol Med Public Heal 2015:4–12.
48. Campos MA, Vargas MA, Regueiro V, Llompart CM, Albertí S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun 72:7107–7114.
49. Spinosa MR, Progida C, Talà A, Cogli L, Alifano P, Bucci C. 2007. The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. Infect Immun 75:3594–3603.
50. Cress BF, Englaender JA, He W, Kasper D, Linhardt RJ, Koffas MAG. 2014. Masquerading microbial pathogens: capsular polysaccharides mimic host-tissue molecules. FEMS Microbiol Rev 38:660–697.
51. Russo TA, Davidson BA, Carlino-MacDonald UB, Helinski JD, Priore RL, Knight PR. 2003. The effects of *Escherichia coli* capsule, O-antigen, host neutrophils, and complement in a rat model of Gram-negative pneumonia. FEMS Microbiol Lett 226:355–361.
52. de Smet AMGA, Kluytmans JAJW, Blok HEM, Mascini EM, Benus RFJ, Bernards AT, Kuijper EJ, Leverstein-van Hall MA, Jansz AR, de Jongh BM, van Asselt GJ, Frenay IHME, Thijsen SFT, Conijn SNM, Kaan JA, Arends JP, Sturm PDJ, Bootsma MCJ, Bonten MJM. 2011. Selective digestive tract decontamination and selective oropharyngeal decontamination and antibiotic resistance in patients in intensive-care units: an open-label, clustered group-randomised, crossover study. Lancet Infect Dis 11:372–380.
53. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Eurosurveillance 19:20939.

54. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006.0008.
55. Zhou L, Lei X, Bochner BR, Wanner BL. 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J Bacteriol 185:4956–4972.
56. McKenzie GJ, Craig NL. 2006. Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn*7* without need for selection of the insertion event. BMC Microbiol 6:39.
57. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2016. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group.
58. Andrews JM. 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48:5–16.
59. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin A V, Sirotkin A V, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477.
60. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644.
61. Treangen TJ, Ondov BD, Koren S, Phillippy AM. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 15:524.
62. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359.
63. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.
64. van Mansfeld R, de Been M, Paganelli F, Yang L, Bonten M, Willems R. 2016. Within-host evolution of the Dutch high-prevalent *Pseudomonas aeruginosa* clone ST406 during chronic colonization of a patient with cystic fibrosis. PLoS One 11:e0158106.
65. Zhou Z, Alikhan N-F, Mohamed K, the Agama Study Group, Achtman M. 2019. The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. Genome Res 30; 138-152.
66. Choi K-H, Schweizer HP. 2006. mini-Tn*7* insertion in bacteria with single *att*Tn*7* sites: example *Pseudomonas aeruginosa*. Nat Protoc 1:153–161.
67. McNally A, Cheng L, Harris SR, Corander J. 2013. The evolutionary path to extraintestinal pathogenic, drug-resistant *Escherichia coli* is marked by drastic reduction in detectable recombination within the core genome. Genome Biol Evol 5:699–710.
68. Malek A, McGlynn K, Taffner S, Fine L, Tesini B, Wang J, Mostafa H, Petry S, Perkins A, Graman P, Hardy D, Pecora N. 2019. Next-generation-sequencing-based hospital outbreak investigation yields insight into *Klebsiella aerogenes* population structure and determinants of carbapenem resistance and pathogenecity. Antimicrob Agents Chemother 63:e02577-18.

Supplemental Materials



Supplemental Figure S1: Maximum specific growth rate of *K. aerogenes* strains. Maximum specific growth rate of the colistin-susceptible, and colistin-resistant *K. aerogenes* strains isolated from patient 37. The values presented represent the means with standard deviations of three independent experiments performed in triplicate. No statistical significant differences were observed between the maximum specific growth rates of the different strains.

Supplemental Table S1: Sequences of oligonucleotide primers used in this study.

Name	Sequence (5'- 3')	Reference
M13 Fwd	TGT AAA ACG ACG GCC AGT	This study
M13 Rev	CAG GAA ACA GCT ATG ACC	This study
BW25113 *glmS* Up	ATA TTC AGT CAA TTA CAA ACA TTA	14
BW25113 *glmS* Down	CGA TCT TCT ACA CCG TTC	14
BasRS sequencing Fwd	AAA GCC CGT ATC CGC AC	14
BasRS sequencing Rev	GAT CTC ACG CAT GAT GTG GC	14
Tn*7*R inward	GAA ATC AGT CCA GTT ATG CTG TG	14
Tn*7*L inward	GGG TGT AGC GTC GTA AGC TAA T	14
BasRS genes Fwd	ATG AAA ATT CTG ATT GTT GAA GA	14
BasRS genes Rev	GTT TAG CGT GCT GGT	This study
BasRS promoter Fwd	CTT CCT CTA CTG CAT CTG GG	14
BasRS promoter Rev	CAC GGT GTT TCC ATC GA	14
BasRS genes overlap Fwd	TGC GCA CTT TGT TCG ATG GAA ACA CCG TGA TGA AAA TTC TGA TTG TTG	This study
BasRS promotor Fwd NotI	TAT CCT GCG GCC GCC TTC CTC TAC TGC ATC TGG G	14
BasRS genes Rev XhoI	TAT CCC CTC GAG GTT TAG CGT GCT GGT GGT	This study
BasRS promotor Fwd NotI short	TAT CCT GCG GCC GCC	14
BasRS genes Rev XhoI short	TAT CCC CTC GAG GTT TA	This study
BasS Δ18 bp restore Fwd	AAA ACG CAT CAG ATT CAA TTA GTT TTC CTC ATT	This study
BasS Δ18 bp restore Rev	CTG CGC CGA CCA ATA TCG CTG CGC CAA CGG CTG ATA TT	This study
BasR E19G Fwd	ACG GCT GAT ATT GAC CAT TGG GGC CAT TT TGT TG	This study
BasR E19G Rev	TGG CGC AGC GAT ATT GGT CGG CG	This study
Tn*7*L	ATT AGC TTA CGA CGC TAC ACC C	40
pGRG36 check with Tn*7*L	ATA TGC ACA GAT GAA AAC GGT G	14

Supplemental Table S2: Strains names, Genbank accession numbers, and species of genomes used in the construction of the phylogenetic tree.[a]

Strain[b]	Genbank assembly accession	Species
42	GCA_000027125.1	*Escherichia coli*
536	GCA_000013305.1	*Escherichia coli*
789	GCA_000819645w.1	*Escherichia coli*
1303	GCA_000829985.1	*Escherichia coli*
6409	GCA_000814145.2	*Escherichia coli*
11128	GCA_000010765.1	*Escherichia coli*
11368	GCA_000091005.1	*Escherichia coli*
12009	GCA_000010745.1	*Escherichia coli*
55989	GCA_000026245.1	*Escherichia coli*
180-PT54	GCA_001650275.1	*Escherichia coli*
2009C-3133	GCA_001420955.1	*Escherichia coli*
2009EL-2050	GCA_000299255.1	*Escherichia coli*
2009EL-2071	GCA_000299475.1	*Escherichia coli*
2011C-3493	GCA_000299455.1	*Escherichia coli*
2011C-3911	GCA_001644725.1	*Escherichia coli*
2012C-4227	GCA_001420935.1	*Escherichia coli*
2013C-4465	GCA_001644745.1	*Escherichia coli*
28RC1	GCA_001612475.1	*Escherichia coli*
644-PT8	GCA_001650295.1	*Escherichia coli*
94-3024	GCA_000801185.2	*Escherichia coli*
ABU 83972	GCA_000148365.1	*Escherichia coli*
ACN001	GCA_001051135.1	*Escherichia coli*
ACN002	GCA_001515725.1	*Escherichia coli*
APEC IMT5155	GCA_000813165.1	*Escherichia coli*
APEC O1	GCA_000014845.1	*Escherichia coli*
APEC O78	GCA_000332755.1	*Escherichia coli*
ATCC 25922	GCA_000743255.1	*Escherichia coli*
ATCC 8739	GCA_000019385.1	*Escherichia coli*
B7A	GCA_000725265.1	*Escherichia coli*
BL21 (TaKaRa)	GCA_000833145.1	*Escherichia coli*
BL21(DE3) #1	GCA_000009565.2	*Escherichia coli*
BL21(DE3) #2	GCA_000022665.2	*Escherichia coli*
BL21-Gold(DE3)pLysS AG	GCA_000023665.1	*Escherichia coli*
C227-11	GCA_000986765.1	*Escherichia coli*
C2566	GCA_001559615.1	*Escherichia coli*
C3026	GCA_001559675.1	*Escherichia coli*
C3029	GCA_001559635.1	*Escherichia coli*
C321.deltaA	GCA_000474035.1	*Escherichia coli*

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C41(DE3)	GCA_000830035.1	*Escherichia coli*
C43(DE3)	GCA_001039415.1	*Escherichia coli*
CB9615	GCA_000025165.1	*Escherichia coli*
CD306	GCA_001513615.1	*Escherichia coli*
CE10	GCA_000227625.1	*Escherichia coli*
CFSAN029787	GCA_001007915.1	*Escherichia coli*
CFT073	GCA_000007445.1	*Escherichia coli*
CI5	GCA_000971615.1	*Escherichia coli*
clone D i14	GCA_000233895.1	*Escherichia coli*
clone D i2	GCA_000233875.1	*Escherichia coli*
CQSW20	GCA_001455385.1	*Escherichia coli*
DH1 #1	GCA_000023365.1	*Escherichia coli*
DH1 #2	GCA_000270105.1	*Escherichia coli*
DH1Ec095	GCA_001183645.1	*Escherichia coli*
DH1Ec104	GCA_001183665.1	*Escherichia coli*
DH1Ec169	GCA_001183685.1	*Escherichia coli*
DHB4	GCA_001559655.1	*Escherichia coli*
E2348/69	GCA_000026545.1	*Escherichia coli*
E24377A	GCA_000017745.1	*Escherichia coli*
EC4115	GCA_000021125.1	*Escherichia coli*
EC958	GCA_000285655.3	*Escherichia coli*
ECC-1470	GCA_000831565.1	*Escherichia coli*
Eco889	GCA_001663475.1	*Escherichia coli*
Ecol_448	GCA_001618365.1	*Escherichia coli*
Ecol_732	GCA_001617565.1	*Escherichia coli*
Ecol_743	GCA_001618325.1	*Escherichia coli*
Ecol_745	GCA_001618345.1	*Escherichia coli*
ECONIH1	GCA_000784925.1	*Escherichia coli*
EDL933	GCA_000732965.1	*Escherichia coli*
ER1821R	GCA_001663075.1	*Escherichia coli*
ER2796	GCA_000800215.1	*Escherichia coli*
ER3413	GCA_000800765.1	*Escherichia coli*
ER3435	GCA_000974885.1	*Escherichia coli*
ER3440	GCA_000974465.1	*Escherichia coli*
ER3445	GCA_000974535.1	*Escherichia coli*
ER3446	GCA_000974825.1	*Escherichia coli*
ER3454	GCA_000974405.1	*Escherichia coli*
ER3466	GCA_000974575.1	*Escherichia coli*
ER3475	GCA_000974865.1	*Escherichia coli*

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ER3476	GCA_000974505.1	*Escherichia coli*
ETEC H10407	GCA_000210475.1	*Escherichia coli*
FRIK2069	GCA_001651925.1	*Escherichia coli*
FRIK2455	GCA_001651965.1	*Escherichia coli*
FRIK2533	GCA_001651945.1	*Escherichia coli*
G749	GCA_001566635.1	*Escherichia coli*
HS	GCA_000017765.1	*Escherichia coli*
HUSEC2011	GCA_000967155.1	*Escherichia coli*
IAI1	GCA_000026265.1	*Escherichia coli*
IAI39	GCA_000026345.1	*Escherichia coli*
IHE3034	GCA_000025745.1	*Escherichia coli*
JEONG-1266	GCA_001558995.2	*Escherichia coli*
JJ1886	GCA_000493755.1	*Escherichia coli*
JJ1887	GCA_001593565.1	*Escherichia coli*
JJ1897	GCA_001513655.1	*Escherichia coli*
JJ2434	GCA_001513635.1	*Escherichia coli*
JW5437-1 substr. MG1655	GCA_001566335.1	*Escherichia coli*
K-12 substr. AG100	GCA_000981485.1	*Escherichia coli*
K-12 substr. BW25113	GCA_000750555.1	*Escherichia coli*
K-12 substr. BW2952	GCA_000022345.1	*Escherichia coli*
K-12 substr. DH10B	GCA_000019425.1	*Escherichia coli*
K-12 substr. GM4792 #1	GCA_001020945.2	*Escherichia coli*
K-12 substr. GM4792 #2	GCA_001021005.2	*Escherichia coli*
K-12 substr. HMS174	GCA_000953515.1	*Escherichia coli*
K-12 substr. MC4100	GCA_000499485.1	*Escherichia coli*
K-12 substr. MDS42	GCA_000350185.1	*Escherichia coli*
K-12 substr. MG1655 #1	GCA_000005845.2	*Escherichia coli*
K-12 substr. MG1655 #2	GCA_000801205.1	*Escherichia coli*
K-12 substr. MG1655 #3	GCA_001308065.1	*Escherichia coli*
K-12 substr. MG1655 #4	GCA_001544635.1	*Escherichia coli*
K-12 substr. MG1655_TMP32XR1	GCA_001308125.1	*Escherichia coli*
K-12 substr. MG1655_TMP32XR2	GCA_001308165.1	*Escherichia coli*
K-12 substr. RV308	GCA_000952955.1	*Escherichia coli*
K-12 substr. W3110	GCA_000010245.1	*Escherichia coli*
KLY	GCA_000725305.1	*Escherichia coli*
KO11	GCA_000147855.3	*Escherichia coli*
KO11FL	GCA_000258025.1	*Escherichia coli*
LF82	GCA_000284495.1	*Escherichia coli*
LY180	GCA_000468515.1	*Escherichia coli*

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MNCRE44	GCA_000931565.1	*Escherichia coli*
MRE600	GCA_001542675.2	*Escherichia coli*
MVAST0167	GCA_001566655.1	*Escherichia coli*
NA114	GCA_000214765.2	*Escherichia coli*
NCM3722	GCA_001043215.1	*Escherichia coli*
NGF1	GCA_001660585.1	*Escherichia coli*
Nissle 1917	GCA_000714595.1	*Escherichia coli*
NRG 857C	GCA_000183345.1	*Escherichia coli*
P12b	GCA_000257275.1	*Escherichia coli*
PCN033	GCA_000219515.3	*Escherichia coli*
PCN061	GCA_001029125.1	*Escherichia coli*
REL606	GCA_000017985.1	*Escherichia coli*
RM12579	GCA_000245515.1	*Escherichia coli*
RM12581	GCA_000671295.1	*Escherichia coli*
RM12761	GCA_000662395.1	*Escherichia coli*
RM13514	GCA_000520035.1	*Escherichia coli*
RM13516	GCA_000520055.1	*Escherichia coli*
RM9387	GCA_000801165.1	*Escherichia coli*
RR1	GCA_001276585.1	*Escherichia coli*
RS218	GCA_000800845.2	*Escherichia coli*
S51	GCA_001660565.1	*Escherichia coli*
S88	GCA_000026285.1	*Escherichia coli*
Sakai substr. RIMD 0509952	GCA_000008865.1	*Escherichia coli*
Sanji	GCA_001610755.1	*Escherichia coli*
Santai	GCA_000827105.1	*Escherichia coli*
SaT040	GCA_001566615.1	*Escherichia coli*
SE11	GCA_000010385.1	*Escherichia coli*
SE15	GCA_000010485.1	*Escherichia coli*
SEC470	GCA_000987875.1	*Escherichia coli*
SF-088	GCA_001280325.1	*Escherichia coli*
SF-166	GCA_001280385.1	*Escherichia coli*
SF-173	GCA_001280405.1	*Escherichia coli*
SF-468	GCA_001280345.1	*Escherichia coli*
SMS-3-5	GCA_000019645.1	*Escherichia coli*
SQ110	GCA_000988425.1	*Escherichia coli*
SQ171	GCA_000988445.1	*Escherichia coli*
SQ2203	GCA_000988465.1	*Escherichia coli*
SQ37	GCA_000988355.1	*Escherichia coli*
SQ88	GCA_000988385.1	*Escherichia coli*

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SRCC 1675	GCA_001612495.1	*Escherichia coli*
SS17	GCA_000730345.1	*Escherichia coli*
SS52	GCA_000803705.1	*Escherichia coli*
ST2747 #1	GCA_000599665.1	*Escherichia coli*
ST2747 #2	GCA_000599685.1	*Escherichia coli*
ST2747 #3	GCA_000599705.1	*Escherichia coli*
ST540 #1	GCA_000597845.1	*Escherichia coli*
ST540 #2	GCA_000599625.1	*Escherichia coli*
ST540 #3	GCA_000599645.1	*Escherichia coli*
ST648	GCA_001485455.1	*Escherichia coli*
TW14359	GCA_000022225.1	*Escherichia coli*
uk_P46212	GCA_001469815.1	*Escherichia coli*
UM146	GCA_000148605.1	*Escherichia coli*
UMNK88	GCA_000212715.2	*Escherichia coli*
UTI89	GCA_000013265.1	*Escherichia coli*
VR50	GCA_000968515.1	*Escherichia coli*
W #1	GCA_000184185.1	*Escherichia coli*
W #2	GCA_000258145.1	*Escherichia coli*
WS4202	GCA_001307215.1	*Escherichia coli*
Xuzhou21	GCA_000262125.1	*Escherichia coli*
YD786	GCA_001442495.1	*Escherichia coli*
ZH063	GCA_001577325.1	*Escherichia coli*
ZH193	GCA_001566675.1	*Escherichia coli*
11E12	GCA_002260745.1	*Klebsiella aerogenes*
1019_EAER	GCA_001053595.1	*Klebsiella aerogenes*
1020_EAER	GCA_001052095.1	*Klebsiella aerogenes*
1277_EAER	GCA_001052565.1	*Klebsiella aerogenes*
1278_EAER	GCA_001054275.1	*Klebsiella aerogenes*
1282_EAER	GCA_001053235.1	*Klebsiella aerogenes*
151_EAER	GCA_001054405.1	*Klebsiella aerogenes*
225_EAER	GCA_001071835.1	*Klebsiella aerogenes*
86_EAER	GCA_001058645.1	*Klebsiella aerogenes*
AR_0007	GCA_002796425.1	*Klebsiella aerogenes*
AR_0009	GCA_002796525.1	*Klebsiella aerogenes*
AR_0018	GCA_002796405.1	*Klebsiella aerogenes*
AR_0062	GCA_002948835.2	*Klebsiella aerogenes*
AR_0161	GCA_003071285.1	*Klebsiella aerogenes*
C10	GCA_001662765.1	*Klebsiella aerogenes*
CAV1320	GCA_001021995.1	*Klebsiella aerogenes*

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CDC UA0804-01	GCA_000755545.1	*Klebsiella aerogenes*
D2	GCA_001662705.1	*Klebsiella aerogenes*
D3	GCA_001662695.1	*Klebsiella aerogenes*
E20	GCA_002946615.1	*Klebsiella aerogenes*
E9	GCA_001662715.1	*Klebsiella aerogenes*
EA1509E	GCA_000334515.1	*Klebsiella aerogenes*
Ea77	GCA_001649605.2	*Klebsiella aerogenes*
FDAARGOS_139	GCA_001593585.2	*Klebsiella aerogenes*
FDAARGOS_152	GCA_001559215.2	*Klebsiella aerogenes*
FDAARGOS_327	GCA_003546885.1	*Klebsiella aerogenes*
FDAARGOS_363	GCA_002591115.1	*Klebsiella aerogenes*
FDAARGOS_513	GCA_003812185.1	*Klebsiella aerogenes*
FGI35	GCA_000383335.1	*Klebsiella aerogenes*
G7	GCA_001571545.2	*Klebsiella aerogenes*
kae_122664	GCA_901484885.1	*Klebsiella aerogenes*
KCTC 2190	GCA_000215745.1	*Klebsiella aerogenes*
MGH 61	GCA_000692155.1	*Klebsiella aerogenes*
MGH 62	GCA_000692175.1	*Klebsiella aerogenes*
MGH 77	GCA_000692195.1	*Klebsiella aerogenes*
MGH 78	GCA_000692215.1	*Klebsiella aerogenes*
MGH174	GCA_002152895.1	*Klebsiella aerogenes*
NCTC10006	GCA_900635435.1	*Klebsiella aerogenes*
NCTC9735	GCA_900637945.1	*Klebsiella aerogenes*
NFIX39	GCA_900119335.1	*Klebsiella aerogenes*
PX01	GCA_002204605.1	*Klebsiella aerogenes*
SCKA020061	GCA_002852865.1	*Klebsiella aerogenes*
UCI 15	GCA_000534335.1	*Klebsiella aerogenes*
UCI 16	GCA_000534315.1	*Klebsiella aerogenes*
UCI 27	GCA_000534255.1	*Klebsiella aerogenes*
UCI 28	GCA_000534235.1	*Klebsiella aerogenes*
UCI 45	GCA_000534135.1	*Klebsiella aerogenes*
UCI 46	GCA_000534115.1	*Klebsiella aerogenes*
UCI 47	GCA_000534095.1	*Klebsiella aerogenes*
UCI 48	GCA_000534075.1	*Klebsiella aerogenes*
UCI119	GCA_002152915.1	*Klebsiella aerogenes*
UCI120	GCA_002152925.1	*Klebsiella aerogenes*
UCI89	GCA_001030125.1	*Klebsiella aerogenes*
UCI90	GCA_001030165.1	*Klebsiella aerogenes*
UCI97	GCA_001030185.1	*Klebsiella aerogenes*

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UMB0295	GCA_002871375.1	*Klebsiella aerogenes*

[a] A total of 178 *E. coli* and 56 *K. aerogenes* genome sequences obtained from NCBI databases were used for construction of phylogenetic trees.
[b] When multiple assemblies had the same strain name, a numerical indicator was added.

Chapter

4

Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak

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Abstract

Klebsiella pneumoniae is emerging as an important nosocomial pathogen due to its rapidly increasing multidrug resistance, which has led to a renewed interest in polymyxin antibiotics, such as colistin, as antibiotics of last resort. However, heteroresistance (i.e., the presence of a subpopulation of resistant bacteria in an otherwise susceptible culture) may hamper the effectiveness of colistin treatment in patients. In a previous study, we showed that colistin resistance among extended-spectrum ß-lactamase (ESBL)-producing *K. pneumoniae* isolates emerged after the introduction of selective digestive tract decontamination (SDD) in an intensive care unit (ICU). In this study, we investigated heteroresistance to colistin among ESBL-producing *K. pneumoniae* isolates by using population analysis profiles. We used whole-genome sequencing (WGS) to identify the mutations that were associated with the emergence of colistin resistance in these *K. pneumoniae* isolates. We found five heteroresistant subpopulations, with minimal inhibitory concentration (MICs) of colistin ranging from 8 to 64 µg/ml, which were derived from five clonally related, colistin-susceptible clinical isolates. WGS revealed the presence of mutations in the *lpxM*, *mgrB*, *phoQ*, and *yciM* genes in colistin-resistant *K. pneumoniae* isolates. In two strains, *mgrB* was inactivated by an IS*3*-like or IS*Kpn14* insertion sequence element. Complementation *in trans* with the wild-type *mgrB* gene resulted in these strains reverting to colistin susceptibility. The MICs for colistin-susceptible strains increased 2- to 4-fold in the presence of the mutated *phoQ*, *lpxM*, and *yciM* alleles. In conclusion, the present study indicates that heteroresistant *K. pneumoniae* subpopulations may be selected for upon exposure to colistin. Mutations in *mgrB* and *phoQ* have previously been associated with colistin resistance, but we provide experimental evidence for roles of mutations in the *yciM* and *lpxM* genes in the emergence of colistin resistance in *K. pneumoniae*.

Introduction

Klebsiella pneumoniae is emerging as an important nosocomial pathogen due to rapidly increasing resistance to practically all currently available antibiotics, in particular carbapenems (1, 2). This Gram-negative opportunistic pathogen can cause wound and urinary tract infections and other life-threatening, hospital-acquired infections, such as pneumonia, bacteremia, and postoperative meningitis (3, 4). Due to increasing multidrug resistance (MDR) among Gram-negative bacteria, including *K. pneumoniae*, and the lack of novel antibiotics to treat infections caused by MDR Gram-negative bacteria (5), there is a renewed interest in the antibiotic colistin as a therapy of last resort (6).

Colistin (polymyxin E) is a cationic polypeptide with a lipid tail which targets anionic lipopolysaccharide (LPS) molecules in the outer membranes of Gram-negative bacteria, introducing changes in the permeability of the membrane which lead to leakage of cell contents and, finally, cell death (7, 8). Resistance to colistin among Gram-negative bacteria in clinical isolates was reported recently (9–11). Several strategies are employed by bacteria to gain resistance to colistin, including LPS modifications, particularly modifications of lipid A, the use of efflux pumps, and overexpression of outer membrane proteins (12). Resistance to colistin in clinical isolates may go undetected when traditional i*n vitro* antibiotic susceptibility testing is used, because of heteroresistance, which denotes the presence of subpopulations of bacterial cells with higher levels of antibiotic resistance than those of the rest of the population in the same culture (13). This phenomenon was described recently for Gram-negative organisms including *Pseudomonas aeruginosa* (14), *Acinetobacter baumannii* (15, 16), and *Enterobacter cloacae* (17).

In a previous study (18), we showed that colistin resistance among extended-spectrum ß-lactamase (ESBL)-producing *K. pneumoniae* isolates emerged after exposure to colistin as part of selective digestive tract decontamination (SDD) in an intensive care unit (ICU), and we postulated that this may be explained by the presence of heteroresistant subpopulations of the colistin-susceptible MDR strains.

In the present study, the existence of colistin-resistant subpopulations among ESBL-producing *K. pneumoniae* isolates was investigated. Through whole-genome sequencing (WGS) and complementation of mutated alleles *in trans*, the roles of mutations in resistance to colistin in *K. pneumoniae* were determined.

Results

Antimicrobial susceptibility and colistin heteroresistance

An overview of the 13 *K. pneumoniae* strains included in this study is listed in Table 1; these strains included eight clinical isolates and five heteroresistant subpopulations. After retesting of antibiotic susceptibilities and confirmation of the presence of the ESBL phenotype by the double-disk synergy test, seven of the eight initially ESBL-producing *K. pneumoniae* clinical isolates were again found to be ESBL positive (isolate 3-CR lost the ESBL phenotype). None of the strains was resistant to carbapenem antibiotics. The six colistin-susceptible isolates had minimal inhibitory concentrations (MICs) of colistin ranging from 1 to 2 µg/ml, and the two colistin-resistant strains had colistin MICs of 16 and 64 µg/ml.

Population analysis profiles (PAPs) revealed the presence of heteroresistance in five clinical isolates (Table 1) (isolates 4-DS, 6-ES, 8-FS, 10-GS, and 12-HS) initially considered colistin-susceptible based on MICs ranging from 1 to 2 µg/ml. Subpopulations of these colistin-heteroresistant isolates grew in the presence of colistin at concentrations of 3 to 10 µg/ml (Figure 1). The MICs for the resistant subpopulations 5-DR, 7-ER, 9-FR, 11-GR, and 13-HR were 32, 48, 16, 8, and 64 µg/ml, respectively (Table 1). The proportion of resistant colonies was on the order of 10^{-6}. The colistin-susceptible reference strain ATCC 700603 survived in the presence of up to 0.5 µg/ml colistin sulfate, and no heteroresistant subpopulations were observed.

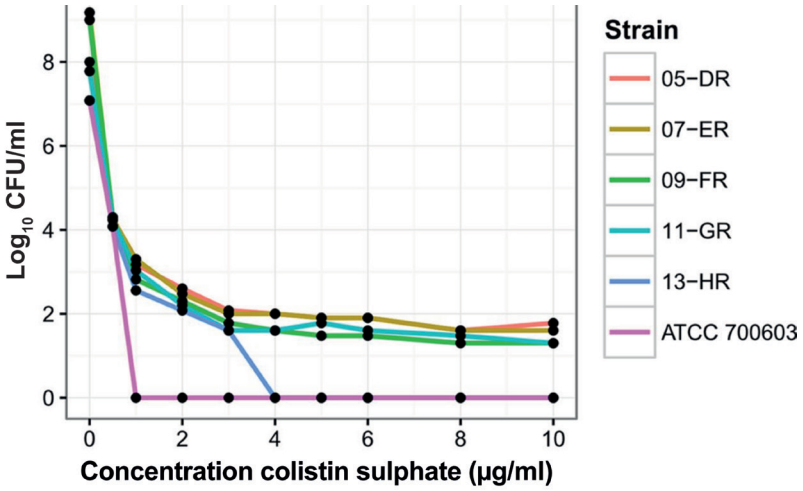

Figure1: Population analysis profiles indicating colistin heteroresistance. Population analysis profiles are shown for five colistin-susceptible isolates after exposure to colistin sulfate. The *y*-axis indicates the number of colonies on Mueller-Hinton agar plates, and concentrations of colistin sulfate are shown on the *x*-axis.

Table 1: Characteristics of ESBL-producing *K. pneumoniae* isolates.[a]

Isolate identifier[a]	Year of isolation	Colistin susceptbility	Clonality (sequence type)[b]	Colistin MIC (µg/ml)[c]	Mutation[d]			
					mgrB	*yciM*	*phoQ*	*lpxM*
1-AS	2002	S	ST43	2				
2-BR	2004	R	ST43	64	IS*3*-like insertion			
3-CR	2007	R	ST1423	16		*Not determined*		
4-DS	2002	S	ST43	1				
5-DR		R		32	IS*Kpn14* insertion			
6-ES	2003	S	ST43	2				
7-ER		R		48		V43G		
8-FS	2003	S	ST43	2				
9-FR		R		16			A21S	
10-GS	2005	S	ST43	2				
11-GR		R		8				V30G
12-HS	2006	S	ST43	4				
13-HR		R		64	4.2 kb deletion			

[a] Isolate identifiers consist of unique numbers used in Figure 1 and Figure 2; letters indicate the code for the patient and whether the strain was susceptible (S) or resistant (R) to colistin. The isolate from patient A was obtained before the introduction of SDD in the ICU, and the remaining seven were obtained thereafter.
[b] Clonality was determined by phylogenetic analysis.
[c] MICs were determined by the broth microdilution method.
[d] The different SNPs, a deletion, and the inactivation of genes due to IS element insertions are indicated in the mutation columns.
Heteroresistant strains are indicated with shading. ST, sequence type.

Phylogenetic analysis of colistin-susceptible and colistin-resistant *K. pneumoniae* isolates

A phylogenetic tree (Figure 2A) for *K. pneumoniae* was generated based on the core genome sequence of 25 publicly available *K. pneumoniae* sequences and the 13 sequenced genomes of the *K. pneumoniae* isolates (Table 1). The core genome consisted of 2637 orthogroups, with a total alignment length of 2,013,123 bp and 209,626 polymorphic sites. The core genome-based phylogenetic tree recapitulated the previously observed population structure of *K. pneumoniae sensu lato*, which includes *Klebsiella quasipneumoniae* (clade KpII) and *Klebsiella variicola* (clade KpIII) (19–21). Seven of the eight clinical isolates from the nosocomial outbreak were closely related to each other and clustered in the *K. pneumoniae* (KpI) clade. A single colistin-resistant isolate (3-CR) was assigned to clade KpIII and therefore appeared to be unrelated to the other isolates from the outbreak. All other strains from the outbreak (seven clinical isolates and five heteroresistant subpopulations) had the same sequence type, i.e., ST43. These data confirm the previously reported existence of an outbreak with closely related *K. pneumoniae* isolates in an ICU (18).

Although the isolates belonged to the same sequence type, a repertoire of distinctly different antibiotic resistance genes was observed (Figure 2B). The isolates carried several antibiotic resistance genes, including aminoglycoside resistance genes and β-lactam resistance genes.

Mutations associated with colistin resistance

Single nucleotide polymorphisms (SNPs) and insertion and deletions (indels) were determined for all paired colistin-susceptible and colistin-resistant isolates. In addition, we determined whether full-length copies of the *mgrB* and *phoQ* genes were present in the isolates, as mutations leading to deletion or inactivation of these genes are a common cause of colistin resistance in *K. pneumoniae* (12, 22–26).

A limited number (1 to 4) of SNPs (see Table S1 in the supplemental material) and indels (see Table S2) distinguished the outbreak isolates. In comparisons of paired colistin-susceptible and colistin-resistant strains originating from the same patients, we identified mutations in *mgrB* that led to disruption of the gene in three colistin-resistant isolates (2-BR, 5-DR, and 13-HR). The event that led to the inactivation of *mgrB* was different for each strain. In strain 2-BR, an IS element (IS*3*-like) was inserted into *mgrB*. In strain 5-DR, the element IS*Kpn14* disrupted *mgrB*. Strain 13-HR had a 4.2-kb deletion including the *mgrB* gene. In all other colistin-resistant strains, *mgrB* was not mutated, meaning that other mutations

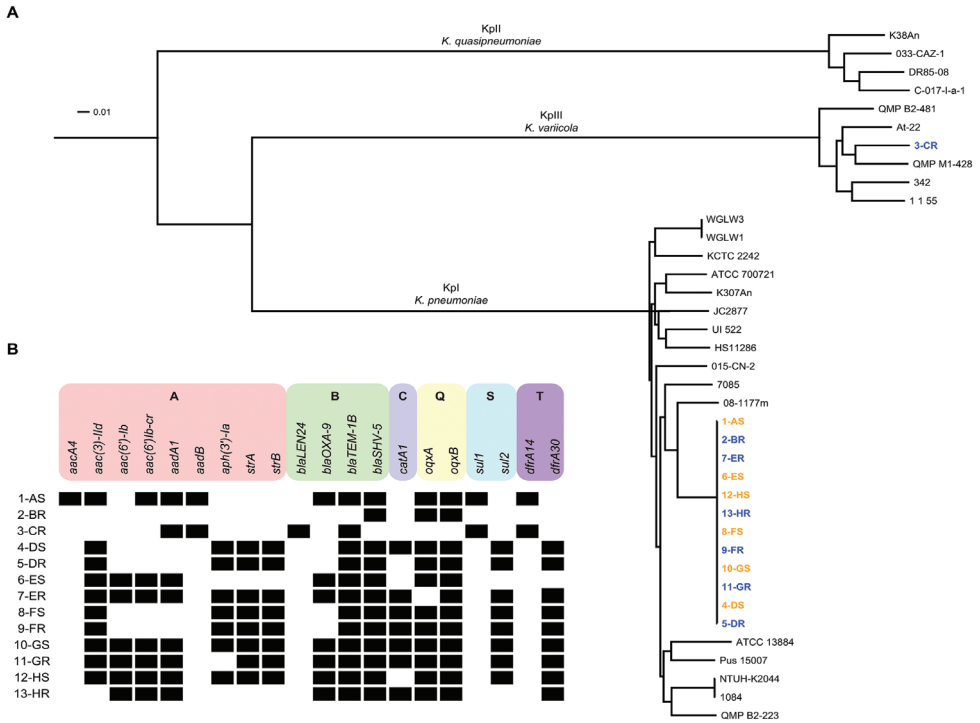

Figure 2: Phylogenetic tree and antibiotic resistance genes of *K. pneumoniae* strains. A) The phylogenetic tree represents a concatenated alignment of 2637 core orthogroups, with a combined length of 2,013,123 bp and 209,626 polymorphic sites, of 38 *K. pneumoniae* strains. The strains sequenced as part of this study are highlighted in color (orange, colistin-susceptible isolates; and blue, colistin-resistant isolates). **B)** Antibiotic resistances detected in the *K. pneumoniae* strains that were sequenced as part of this study. Classes of antibiotic resistance genes are indicated as follows: A, aminoglycoside resistance genes; B, β-lactam resistance genes; C, chloramphenicol resistance genes; Q, quinolone resistance genes; S, sulfonamide resistance genes; and T, trimethoprim resistance genes.

must have led to the colistin resistance phenotypes of 7-ER, 9-FR, and 11-GR.

Interestingly, in these isolates, nonsynonymous SNPs were identified in genes that had predicted roles in outer membrane biosynthesis. Since the outer membrane is the main target of colistin, mutations in genes involving outer membrane biosynthesis may contribute to colistin resistance. In the strains from patient E, the only SNP difference between the colistin-susceptible (6-ES) and colistin-resistant (7-ER) isolates was a nonsynonymous SNP causing a V43G amino acid substitution encoded within the *yciM* gene. A mutation in *phoQ*, resulting in an A21S amino acid change, was identified in the colistin-resistant isolate 9-FR. The colistin-susceptible and colistin-resistant strains from patient G (10-GS and 11-GR, respectively) differed from each other by only 2 SNPs. One of these SNPs mapped to the *lpxM* gene, causing a V30G substitution.

Mutations in *mgrB*, *yciM*, *phoQ*, and *lpxM* contribute to colistin resistance

To verify whether the IS element insertions and amino acid substitutions identified by WGS contributed to colistin resistance, the colistin-susceptible and -resistant strain pairs were complemented *in trans* with the wild-type and mutated genes. Transformation of the strains with a control plasmid containing the *lacZ* gene did not alter the MIC of colistin for any strain. Complementation *in trans* with plasmids harboring the parental *mgrB* gene resulted in a reversal toward colistin susceptibility in isolates 2-BR and 5-DR (Figure 3). Complementation *in trans* for the 12-HS and 13-HR pair was not performed due to difficulties in cloning the 4.2-kb deleted region spanning *mgrB*. Complementation with the mutated forms of *phoQ*, *yciM*, and *lpxM* resulted in decreased susceptibility to colistin. Electrotransformation of the colistin-susceptible strain 6-ES with a plasmid containing the mutated *yciM* gene resulted in a 3-fold increase in the MIC of colistin. The *phoQ* and *lpxM* mutations resulted in a 4-fold increased MIC of colistin for strain 8-FS and a 2-fold increased MIC of colistin for strain 10-GS, respectively. These observations indicate that mutations in *yciM*, *phoQ*, and *lpxM* are dominant when present *in trans* with the corresponding wild-type alleles, resulting in a colistin resistance phenotype. Because the deletions and inactivations of *mgrB* are loss-of-function mutations, they are recessive in the presence of the intact *mgrB* gene.

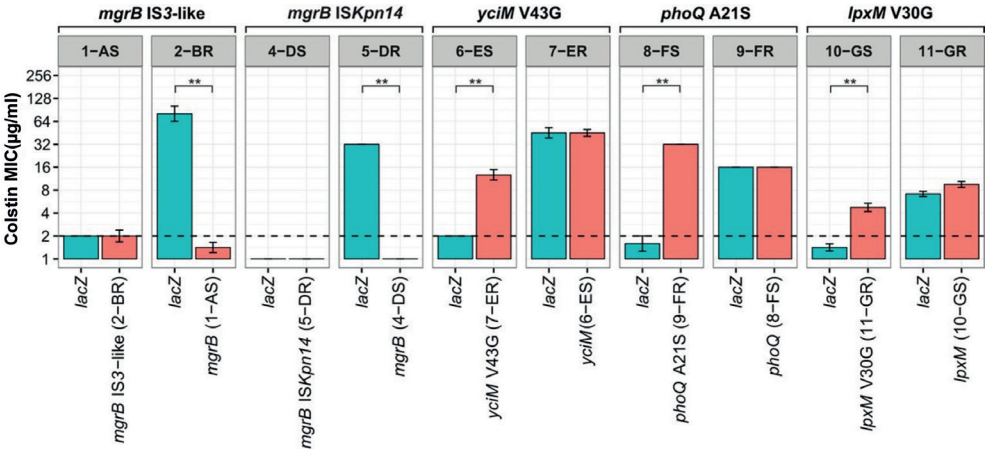

Figure3: Experimental validation of the roles of identified mutations in colistin resistance. Wild-type and mutated *mgrB*, *yciM*, *phoQ*, and *lpxM* alleles were cloned into corresponding *K. pneumoniae* strains by use of a PCR-TRAP cloning system (red). The strains from which the corresponding genes originated are indicated in parentheses. A vector containing a gene encoding the LacZ α-peptide of *E. coli* was used as a vector control (blue). The colistin MICs for the electrotransformed *K. pneumoniae* strains were determined by reference broth microdilution testing using cation-adjusted Mueller-Hinton broth supplemented with 10 µg/ml tetracycline. The colistin MIC resistance breakpoint (i.e., 2 µg/ml) is indicated with a black dashed line. Significant differences ($P<0.05$; Mann-Whitney test) are indicated by double asterisks. The y-axis was plotted on a $\log_2$ scale.

Discussion

In a previous study, we showed that prolonged use of colistin as part of SDD in an outbreak setting resulted in the emergence of colistin resistance among ESBL-producing *K. pneumoniae* clinical isolates (18). The main finding of the present follow-up study is that heteroresistance among these apparently susceptible isolates forms a reservoir for the emergence of colistin resistance during treatment.

Heteroresistance has been recognized in both Gram-positive and Gram-negative bacteria and is a phenomenon where subpopulations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic (13). Heteroresistance can be intrinsic or acquired. Intrinsic heteroresistance occurs without pre-exposure to the antibiotic, but heteroresistance may also be acquired or induced after initial exposure to antibiotics (13). Heteroresistance may have an impact on the outcome of clinical infection, particularly because its detection may be difficult by routine microbiology susceptibility testing (27). The PAP method used in the present study is considered the gold standard for determining heteroresistance (13). Phylogenetic analysis confirmed the clonality of all clinical isolates (excluding 3-CR). However, the absence of overlapping SNPs between clonal colistin-resistant isolates, isolated from different patients over a time span of 4 years, argues in favor of acquired, *de novo* resistance in individual strains under SDD use, rather than selection of preexisting mutants or transmission of the resistant strains between patients.

Although heteroresistance has previously been described for *K. pneumoniae* (28, 29), data on the molecular basis of colistin resistance in this species are scarce. Studies have recently shown that mutations in the genes encoding the PhoPQ two-component system and inactivation of the *mgrB* gene are important pathways by which *K. pneumoniae* can acquire resistance to colistin (30). In the present study, mutations in *phoP*Q and *mgrB* were found in four of the six analyzed isolates.

Mutations in the *phoQ* gene are a common mechanism by which Gram-negative bacteria, including *K. pneumoniae*, gain resistance to colistin (12, 25, 26). PhoQ is a sensor histidine kinase which, together with its cognate response regulator, PhoP, forms a two-component system (2CS). PhoPQ is activated under a variety of conditions, including low pH, low concentrations of Mg^{2+}, and the presence of antimicrobial peptides, including colistin. Activation of PhoPQ leads to the expression of genes that modify LPS in a variety of ways, including deacylation of lipid A or modification of lipid A by 4-amino-4-deoxy-L-arabinose, leading to colistin resistance (12). We found that a mutation in *phoQ* resulting in an amino acid

change (A21S) in the sensor domain of PhoQ which leads to colistin resistance was dominant over the nonmutated copy of *phoQ*. Notably, a mutation in *Salmonella phoQ*, resulting in a threonine-to-isoleucine change at position 48, in the sensor domain of the PhoQ protein, was also found to be dominant, as it constitutively increased phosphorylation of the response regulator PhoP (31, 32). A similar mechanism may explain why the *phoQ* mutation of *Klebsiella pneumoniae* strain 9-FR is dominant. Several studies have recently shown that inactivation of the *mgrB* gene, which encodes a negative regulator of the 2CS PhoPQ, causes colistin resistance (23–26). The inactivation or deletion of *mgrB* leads to higher activity of PhoPQ, which in turn activates the *pmrHFIJKLM* operon, which is responsible for modification of lipid A.

The *mgrB* and *phoP*Q genes were not mutated in the remaining two colistin-resistant isolates. In the heteroresistant strain from patient E, a mutation leading to an amino acid substitution encoded within the *yciM* gene was found. In *Escherichia coli*, *yciM* contributes to cell wall integrity by regulating LPS biosynthesis (33, 34), and a deletion in *yciM* leads to decreased susceptibility to colistin (35). It is possible that the mutation in *yciM* in *K. pneumoniae* increases LPS production, leading to higher levels of LPS in the outer membrane, which could titrate out the destabilizing effect of colistin binding to LPS. In the heteroresistant strain from patient G, a nonsynonymous mutation was found in the *lpxM* gene. LpxM is responsible for the addition of one of the secondary acyl chains to lipid A in Enterobacteriaceae (36, 37). In *K. pneumoniae*, deletion of *lpxM* contributes to susceptibility to antimicrobial peptides, including colistin (38). It is possible that the mutation in *lpxM* alters the acylation of lipid A, thereby making the strain more resistant to colistin. To our knowledge, this is the first time that mutations in *yciM* and *lpxM* have been found in *K. pneumoniae* and linked to reduced susceptibility to colistin. Currently, we cannot mechanistically explain why the mutated alleles of *yciM* and *lpxM* are dominant over the wild-type alleles. Conceivably, the presence of these alleles may interfere with the complex regulation of LPS biosynthesis in *Klebsiella* (39).

The present study shows that heteroresistance to colistin is present in a clonal population of ESBL-producing *K. pneumoniae* strains that were isolated from ICU patients who had been exposed to colistin. Our study highlights the multiple evolutionary trajectories that can lead to colistin resistance in *K. pneumoniae* and underscores the importance of monitoring the existence of colistin-resistant subpopulations in diagnostic susceptibility testing of *K. pneumoniae*.

Materials and Methods

Clinical data and bacterial isolates

K. pneumoniae isolates were collected during a study on the emergence of colistin resistance in Enterobacteriaceae before and after the introduction of SDD in an ICU (18). Briefly, SDD, a topical mixture of antibiotics, including tobramycin, colistin, and amphotericin B at doses (given four to eight times daily) of 80, 100, and 500 mg, respectively, was introduced in 2002 to control an outbreak of ESBL-producing *K. pneumoniae* in an ICU. Reexamination of stored isolates from surveillance and clinical cultures from ICU patients before and after the start of SDD revealed that all tested isolates obtained before the start of SDD were colistin-susceptible, whereas 71% of isolates from cultures obtained thereafter were resistant. Molecular typing of the isolates revealed that most of them were clonally related (18).

In this study, we included a total of 13 strains: eight genetically related ESBL-producing *K. pneumoniae* clinical isolates (one isolate per patient) with known colistin MICs and five heteroresistant subpopulations of these isolates. Genetic relatedness was determined by use of the DiversiLab system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The eight clinical isolates included six colistin-susceptible isolates, one of which was obtained before the start of SDD and five thereafter, and two colistin-resistant isolates obtained after the start of SDD. The six colistin-susceptible isolates and one of the two colistin-resistant isolates were genotypically identical based on DiversiLab typing.

K. pneumoniae ATCC 700603 (ATCC, Manassas, VA) was included as a colistin-susceptible reference strain. The identities of all isolates were confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) analysis according to the manufacturer's instructions, and strains were stored at –80°C before the investigations described in this study.

Antibiotic susceptibility testing

Routine antimicrobial susceptibility testing was performed by use of a Vitek 2 Advanced Expert system and Etest (bioMérieux, Marcy l'Etoile, France), using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org/clinical_breakpoints). The presence of ESBLs

was determined with the double-disk synergy test (40). The MICs of colistin against the *K. pneumoniae* strains were determined by broth microdilution testing (http://www.eucast.org/guidance_documents) using cation-adjusted Mueller-Hinton broth (MHCB). Determination of the colistin MICs of electrotransformed strains was performed with MHCB supplemented with 10 µg/ml tetracycline.

PAPs

To investigate the presence of colistin heteroresistance, PAPs were determined for two replicates by spiral plating 50 µl aliquots of the starting bacterial cell suspension (corresponding to a 0.5 McFarland standard for *K. pneumoniae* cultures grown on blood agar plates for 24 h at 37°C; approximately 10^8 CFU/ml) on Mueller-Hinton agar plates with or without colistin sulfate (0.5, 1, 2, 3, 4, 5, 6, 8, and 10 µg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands) as described by Li *et al.* (41). After 24 h of incubation at 37°C, the number of colonies was counted. Colistin heteroresistance was defined as the presence of a colistin-susceptible isolate with a colistin MIC of < 2 µg/ml in which detectable colistin-resistant subpopulations were able to grow in the presence of ≥ 2 µg/ml colistin (41). The detection limit of colistin-resistant subpopulations was 20 CFU/ml.

Genome sequencing and assembly

Genomic DNAs of *K. pneumoniae* isolates were isolated from overnight cultures grown in Luria broth at 37°C with shaking at 250 rpm by use of a Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Sequence libraries were prepared with a Nextera XT kit (Illumina, San Diego, CA) used according to the manufacturer's instructions. Libraries were sequenced on an Illumina MiSeq system with a 500-cycle (2 × 250 bp) MiSeq reagent kit v2. High-throughput sequence (HTS) data were analyzed for quality with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and raw 2 x 250 bp paired-end reads were filtered with Nesoni 0.109 (http://github.com/Victorian-Bioinformatics-Consortium/nesoni). *De novo* genome assembly was performed with SPAdes 2.5.1 (42), with k-mers 25, 35, 45, 57, and 69, using the following cutoffs for the minimum contig/scaffold: a size of 500 bp and average nucleotide coverage (10-fold).

Phylogenetic analysis

Publicly available WGS sequence data for 25 *K. pneumoniae* strains were downloaded from the NCBI databases in February 2016. The strains used in phylogenetic analysis were selected to cover all *K. pneumoniae* clades, as previously determined by Holt *et al.* (19). For strains for which only raw sequence reads were available, assemblies were generated with SPAdes 2.5.1 (42), as described above. To ensure consistent gene prediction and annotation of all 38 genomes in this study, all genome sequences were reannotated with Prokka v1.10, using the default settings (43). To identify the core genome of these strains, first an all-against-all protein BLAST sequence similarity search of annotated and translated gene sequences was performed with default settings, except for an E-value of 1e–05. Based on the protein BLAST output, orthologous groups were determined and clustered using OrthAgogue v1.0.3 (44) (settings -u and -o 50) and MCL v14-137 (45) (settings -I 1.5), respectively. The nucleotide sequences of orthologous groups containing exactly one representative protein from each of the *K. pneumoniae* genomes were extracted and then aligned using MUSCLE v3.8.31 (46). Gaps were removed from each alignment by using trimAl v1.6 (47), resulting in alignments of equal length (core genome alignments) which were then concatenated. Subsequently, Parsnp v1.2 (48) (settings -r !, -c, and -C 1000) was used to construct a maximum likelihood phylogenetic tree from the variable positions in these core genome alignments. The tree was midpoint rooted and visualized using FigTree software (v1.4.2; http://tree.bio.ed.ac.uk/software/figtree).

Identification of SNPs and indels

Mapping of the Nesoni-filtered reads against the complete genome sequence of *K. pneumoniae* MGH 78578 (NCBI accession number NC_009648) was performed with Bowtie2 v2.2.0 (49) (settings -X 1200, and -a). Genomic repeats were removed from the analyses by filtering out reads that mapped to multiple positions in the *K. pneumoniae* MGH 78578 genome. To call SNP and indels, SAMtools 0.1.18 (50) was used with the following settings: Q score of ≥50, mapping quality of ≥30, mapping depth of ≥10 reads, consensus of ≥75% to support a call, and ≥1 supporting reads in each direction.

Multilocus sequence typing (MLST) and identification of antibiotic resistance genes

Sequence types of the isolates were determined by submitting the genome assemblies to MLST, version 1.8 (51). Antibiotic resistance genes in the genome assemblies were identified by ResFinder v2.1 (52).

Complementation *in trans*

The genes that were mutated in the colistin-resistant *K. pneumoniae* strains were amplified from both the susceptible and resistant strains by PCR using 2× Phusion HF master mix (Thermo Scientific, Landsmeer, the Netherlands) and the primers listed in Table S3 in the supplemental material. The amplified fragments were cloned into the PCR-TRAP cloning system (GenHunter, Nashville, TN), and the resulting plasmids (encoding resistance to tetracycline) were transformed into electrocompetent colistin-susceptible or -resistant *K. pneumoniae* strains by electroporation. The cloned amplicons were sequenced to ensure the absence of errors introduced during PCR (Macrogen Europe, Amsterdam, the Netherlands). Transformants were selected by overnight incubation at 37°C on Luria agar supplemented with 10 µg/ml of tetracycline. The *lacZ* gene fragment encoding the LacZ α-peptide was used as a control insert.

Accession number(s)

Sequence data from this study were deposited in NCBI's Short Read Archive (SRA) under accession number SRA354747.

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References

1. Lee C-R, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. 2016. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: epidemiology, genetic context, treatment options, and detection methods. Front Microbiol 7:895.
2. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP. 2013. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infect Dis 13:785–796.
3. Podschun R, Ullmann U. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11:589–603.
4. Brisse S, Fevre C, Passet V, Issenhuth-Jeanjean S, Tournebize R, Diancourt L, Grimont P. 2009. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. PLoS One 4:e4982.
5. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48:1–12.
6. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. Clin Infect Dis 40:1333–1341.
7. Hancock RE. 1997. Peptide antibiotics. Lancet 349:418–422.
8. Landman D, Georgescu C, Martin DA, Quale J. 2008. Polymyxins revisited. Clin Microbiol Rev 21:449–465.
9. Giacobbe DR, Del Bono V, Trecarichi EM, De Rosa FG, Giannella M, Bassetti M, Bartoloni A, Losito AR, Corcione S, Bartoletti M, Mantengoli E, Saffioti C, Pagani N, Tedeschi S, Spanu T, Rossolini GM, Marchese A, Ambretti S, Cauda R, Viale P, Viscoli C, Tumbarello M, ISGRI-SITA (Italian Study Group on Resistant Infections of the Società Italiana Terapia Antinfettiva). 2015. Risk factors for bloodstream infections due to colistin-resistant KPC-producing *Klebsiella pneumoniae*: results from a multicenter case-control-control study. Clin Microbiol Infect 21:1106.e1-1106.e8.
10. Weterings V, Zhou K, Rossen JW, van Stenis D, Thewessen E, Kluytmans J, Veenemans J. 2015. An outbreak of colistin-resistant *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* in the Netherlands (July to December 2013), with inter-institutional spread. Eur J Clin Microbiol Infect Dis 34:1647–1655.
11. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Eurosurveillance 19:20939.
12. Olaitan AO, Morand S, Rolain J-M. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643.
13. El-Halfawy OM, Valvano MA. 2015. Antimicrobial heteroresistance: an emerging field in need of clarity. Clin Microbiol Rev 28:191–207.
14. Hermes DM, Pormann Pitt C, Lutz L, Teixeira AB, Ribeiro VB, Netto B, Martins AF, Zavascki AP, Barth AL. 2013. Evaluation of heteroresistance to polymyxin B among carbapenem-susceptible and -resistant *Pseudomonas aeruginosa*. J Med Microbiol 62:1184–1189.
15. Hawley JS, Murray CK, Jorgensen JH. 2008. Colistin heteroresistance in *Acinetobacter* and its association with previous colistin therapy. Antimicrob Agents Chemother 52:351–352.
16. Rodríguez CH, Barberis C, Nastro M, Bombicino K, Granados G, Vay C, Famiglietti A. 2012. Impact of heteroresistance to colistin in meningitis caused by *Acinetobacter* baumannii. J Infect 64:119–121.
17. Napier BA, Band V, Burd EM, Weiss DS. 2014. Colistin heteroresistance in *Enterobacter cloacae* is associated with cross-resistance to the host antimicrobial lysozyme. Antimicrob Agents Chemother 58:5594–5597.
18. Halaby T, al Naiemi N, Kluytmans J, van der Palen J, Vandenbroucke-Grauls CMJE. 2013. Emergence of colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. Antimicrob Agents Chemother 57:3224–3229.
19. Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, Jenney A, Connor TR, Hsu LY, Severin J, Brisse S, Cao H, Wilksch J, Gorrie C, Schultz MB, Edwards DJ, Nguyen K V, Nguyen TV, Dao TT, Mensink M, Minh VL, Nhu NTK, Schultsz C, Kuntaman K, Newton PN, Moore CE, Strugnell RA, Thomson NR. 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc Natl Acad Sci U S A 112:E3574–E3581.

20. Brisse S, Verhoef J. 2001. Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* genes sequencing and automated ribotyping. Int J Syst Evol Microbiol 51:915–924.
21. Bialek-Davenet S, Criscuolo A, Ailloud F, Passet V, Jones L, Delannoy-Vieillard A-S, Garin B, Hello S Le, Arlet G, Nicolas-Chanoine M-H, Decré D, Brisse S. 2014. Genomic definition of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* clonal groups. Emerg Infect Dis 20:1812–1820.
22. Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. Antimicrob Agents Chemother 57:5521–5526.
23. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, the COLGRIT Study Group, Vatopoulos A, Rossolini GM. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob Agents Chemother 58:5696–5703.
24. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Türkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. J Antimicrob Chemother 70:75–80.
25. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Chaisiri K, Komalamisra C, Adelowo OO, Fagade OE, Banjo OA, Oke AJ, Adler A, Assous MV, Morand S, Raoult D, Rolain JM. 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. Int J Antimicrob Agents 44:500–507.
26. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adamsa MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. Antimicrob Agents Chemother 59:536–543.
27. Charles PGP, Ward PB, Johnson PDR, Howden BP, Grayson ML. 2004. Clinical features associated with bacteremia due to heterogeneous vancomycin-intermediate *Staphylococcus aureus*. Clin Infect Dis 38:448–451.
28. Bogdanovich T, Adams-Haduch JM, Tian G-B, Nguyen MH, Kwak EJ, Muto C a, Doi Y. 2011. Colistin-resistant, *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae* belonging to the international epidemic clone ST258. Clin Infect Dis 53:373–376.
29. Meletis G, Tzampaz E, Sianou E, Tzavaras I, Sofianou D. 2011. Colistin heteroresistance in carbapenemase-producing *Klebsiella pneumoniae*. J Antimicrob Chemother 66:946–947.
30. Jayol A, Nordmann P, Brink A, Poirel L. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. Antimicrob Agents Chemother 59:2780–2784.
31. Gunn JS, Hohmann EL, Miller SI. 1996. Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. J Bacteriol 178:6369–6373.
32. Baud D, Benyacoub J, Revaz V, Kok M, Ponci F, Bobst M, Curtiss R, De Grandi P, Nardelli-Haefliger D. 2004. Immunogenicity against human papillomavirus type 16 virus-like particles is strongly enhanced by the PhoPc phenotype in *Salmonella enterica* serovar Typhimurium. Infect Immun 72:750–756.
33. Mahalakshmi S, Sunayana MR, Saisree L, Reddy M. 2014. *yciM* is an essential gene required for regulation of lipopolysaccharide synthesis in *Escherichia coli*. Mol Microbiol 91:145–157.
34. Nicolaes V, El Hajjaji H, Davis RM, Van der Henst C, Depuydt M, Leverrier P, Aertsen A, Haufroid V, Ollagnier de Choudens S, De Bolle X, Ruiz N, Collet J-F. 2014. Insights into the function of YciM, a heat shock membrane protein required to maintain envelope integrity in *Escherichia coli*. J Bacteriol 196:300–309.
35. Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, Tran K, Miller JH. 2010. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother 54:1393–1403.
36. Somerville JE, Cassiano L, Bainbridge B, Cunningham MD, Darveau RP. 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. J Clin Invest 97:359–365.
37. Khan SA, Everest P, Servos S, Foxwell N, Zähringer U, Brade H, Rietschel ET, Dougan G, Charles IG, Maskell DJ. 1998. A lethal role for lipid A in *Salmonella* infections. Mol Microbiol 29:571–579.
38. Clements A, Tull D, Jenney AW, Farn JL, Kim S-H, Bishop RE, McPhee JB, Hancock REW, Hartland EL,

Pearse MJ, Wijburg OLC, Jackson DC, McConville MJ, Strugnell RA. 2007. Secondary acylation of *Klebsiella pneumoniae* lipopolysaccharide contributes to sensitivity to antibacterial peptides. J Biol Chem 282:15569–15577.

39. De Majumdar S, Yu J, Fookes M, McAteer SP, Llobet E, Finn S, Spence S, Monaghan A, Kissenpfennig A, Ingram RJ, Bengoechea J, Gally DL, Fanning S, Elborn JS, Schneiders T. 2015. Elucidation of the RamA regulon in *Klebsiella pneumoniae* reveals a role in LPS regulation. PLoS Pathog 11:e1004627.
40. Livermore DM, Brown DF. 2001. Detection of beta-lactamase-mediated resistance. J Antimicrob Chemother 48 (Suppl:S59–S64.
41. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L. 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter* baumannii. Antimicrob Agents Chemother 50:2946–2950.
42. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin A V, Sirotkin A V, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477.
43. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.
44. Ekseth OK, Kuiper M, Mironov V. 2014. orthAgogue: an agile tool for the rapid prediction of orthology relations. Bioinformatics 30:734–736.
45. Van Dongen S. 2008. Graph clustering via a discrete uncoupling process. SIAM J Matrix Anal Appl 30:121–141.
46. Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5:113.
47. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25:1972–1973.
48. Treangen TJ, Ondov BD, Koren S, Phillippy AM. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 15:524.
49. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359.
50. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.
51. Larsen M V., Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Pontén T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol 50:1355–1361.
52. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644.

Supplemental Materials

Table S1. Single nucleotide polymorphisms.[a]

Annotation	Gene length (nt)	Mutation position (nt)
Bifunctional polymyxin resistance protein ArnA	1986	1626
Cation efflux system protein CusC precursor	1386	288
Cobalt import ATP-binding protein CbiO	825	169
Divalent metal cation transporter MntH	1242	1062
DNA polymerase II	2358	2177
Glutamine transport ATP-binding protein GlnQ	780	417
GMP/IMP nucleotidase YrfG	684	284
HTH-type transcriptional regulator AscG	1014	680
HTH-type transcriptional regulator MalT	2706	1815
Hypothetical protein	369	92
Hypothetical protein	1062	193
Lipid A biosynthesis $(KDO)_2$-(lauroyl)-lipid IV(A) acyltransferase LpxM	1071	887
Lipopolysaccharide export system permease protein LptG	1083	330
Maltose transport system permease protein MalF	1308	181
Metallo-beta-lactamase superfamily protein	852	286
NADH oxidase	2034	2016
Outer membrane usher protein FimD precursor	2613	952
Putative arginine/ornithine antiporter YdgI	1383	386
Putative ATP-dependent helicase DinG	1911	1612
Putative cyclic-di-GMP phosphodiesterase AdrB	1563	140
Putative hydroxypyruvate reductase YdgI	1260	1038
Putative lipoprotein ChiQ precursor	333	296
Replicative DNA helicase	1416	596
Ribosomal protein S12 methylthiotransferase accessory factor YcaO	1761	4
Sensor protein PhoQ	1467	1404
SpoVR family protein	1533	84
Tetratricopeptide repeat protein YciM	1170	128
Transcriptional regulator LsrR	942	818
Transketolase 2	1995	809

[a] Isolate identifiers consist of unique numbers used in Figures 1 and 2; letters: code of the patient, and whether the strain was susceptible (S) or resistant (R) to colistin. Isolate from patient A was obtained before the start of SDD, the remaining five colistin-susceptible isolates thereafter. Synonymous mutations are in regulator font, non-synonymous mutations are in bold. Proteins of interest are indicated with shading.

Supplemental Table S1 continued from page 117

1-AS	2-BR	4-DS	5-DR	6-ES
	GAC[D] > GAT[D]			
				TAT[Y] > CAT[H]
CTG[L] > ATG[M]				
	CAC[H] > AAC[N]			**CAC[H] > AAC[N]**
	GCG[A] > GTG[V]			
			TAC[Y] > TTC[F]	
CCA[P] > CCG[P]	CCA[P] > CCG[P]		CCA[P] > CCG[P]	CCA[P] > CCG[P]
				AGC[S] > TGC[C]
CTT[L] > CTG[L]	CTT[L] > CTG[L]			CTT[L] > CTG[L]
	CAG[Q] > CTG[L]			
	GTG[V] > GGG[G]			
ATC[I] > AGC[S]				
			GCC[A] > GGC[G]	
	TTC[F] > GTC[V]			**TTC[F] > GTC[V]**

Supplemental Table S1 continued from page 118

7-ER	8-FS	9-FR	10-GS	11-GR
TAT[Y] > CAT[H]				
			GAT[D] > GAG[E]	
CAC[H] > AAC[N]	**CAC[H] > AAC[N]**	**CAC[H] > AAC[N]**	**CAC[H] > AAC[N]**	**CAC[H] > AAC[N]**
		TCG[S] > TTG[L]		
CCA[P] > CCG[P]	CCA[P] > CCG[P]	CCA[P] > CCG[P]	CCA[P] > CCG[P]	CCA[P] > CCG[P]
				GTC[V] > GGC[G]
AGC[S] > TGC[C]				
		CGT[R] > AGT[S]		
			ATT[I] > ATA[I]	
CTT[L] > CTG[L]	CTT[L] > CTG[L]	CTT[L] > CTG[L]	CTT[L] > CTG[L]	CTT[L] > CTG[L]
		GAT[D] > TAT[Y]		
		GCC[A] > TCC[S]		
TTC[F] > GTC[V]	**TTC[F] > GTC[V]**	**TTC[F] > GTC[V]**	**TTC[F] > GTC[V]**	**TTC[F] > GTC[V]**
GTT[V] > GGT[G]				
			TGC[C] > TTC[F]	**TGC[C] > TTC[F]**
		GCG[A] > GTG[V]		

Supplemental Table S1 continued from page 119

12-HS	13-HR
CAG[Q] > CTG[L]	**CAG[Q] > CTG[L]**
CAC[H] > AAC[N]	**CAC[H] > AAC[N]**
	CTG[L] > TTG[M]
CCA[P] > CCG[P]	CCA[P] > CCG[P]
GCC[A] > ACC[T]	
CTT[L] > CTG[L]	CTT[L] > CTG[L]
TGG[W] > TGA[stop]	
AAA[K] > AAT[N]	**AAA[K] > AAT[N]**
TTC[F] > GTC[V]	**TTC[F] > GTC[V]**
TGC[C] > TTC[F]	**TGC[C] > TTC[F]**

Table S2: Nucleotide insertions and deletions.[a]

Annotation	Gene length (nt)	Mutation position (nt)	1-AS	2-BR	4-DS	5-DR	6-ES
Divalent metal cation transporter MntH	1242	1027		GCCCC > GCCC (-1C)			GCCCC > GCCC (-1C)
DNA translocase FtsK	1872	4		G > GC (+1C)		G > GC (+1C)	
DNA translocase FtsK	2385	2385		G > GC (+1C)		G > GC (+1C)	
General stress protein 69	897	868				A > AC (+1C)	
Hypothetical protein	180	109				C > CG (+1G)	
Putative assembly protein	1848	385	GC > GCC (+1C)				
Putative ATP-dependent transporter SufC	747	295					G > GA (+1A)
Signal transduction histidine-protein kinase BarA	2751	1012					

7-ER	8-FS	9-FR	10-GS	11-GR	12-HS	13-HR
	GCCCC > GCCC (-1C)	GCCCC > GCCC (-1C)	GCCCC > GCCC (-1C)	GCCCC > GCCC (-1C)	GCCCC > GCCC (-1C)	GCCCC > GCCC (-1C)
GCCCC > GCCC (-1C)	G > GC (+1C)		G > GC (+1C)	G > GC (+1C)		G > GC (+1C)
	G > GC (+1C)		G > GC (+1C)	G > GC (+1C)		G > GC (+1C)
	CC > CGC (+1G)			C > CG (+1G)		
	G > GA (+1A)	G > GA (+1A)	G > GA (+1A)	G > GA (+1A)		
			GC > G (-1C)	GC > G (-1C)	GC > G (-1C)	

[a] Isolate identifiers consist of unique numbers used in Figures 1 and 2; letters: code of the patient, and whether the strain was susceptible (S) or resistant (R) to colistin. Isolate from patient A was obtained before the start of SDD, the remaining five colistin-susceptible isolates thereafter. Mutation is indicated between brackets.

Supplemental Table S2 continued

Table S3: PCR amplification primers used in this study

Name	Sequence (5′- 3′)	Gene	Reference
12-*mgrB*-Fwd	AAC CAT AAC AAC AGA CCG AC	*mgrB*	This study
12-*mgrB*-Rev	ATC CCT GGC TTG ATT TTG AC	*mgrB*	
45-*mgrB*-Fwd	ATA ACA ACA GAC CGA CAA GC	*mgrB*	
45-*mgrB*-Rev	TTC GTA ATA CAG TTA GCC GC	*mgrB*	
67-*yciM*-Fwd	AAA ACG GAT GCC TG AAG C	*yciM*	
67-*yciM*-Rev	GTT GTA ACT AAC GGA GGG C	*yciM*	
89-*phoQ*-Fwd	GGC GTT TAC TGA AAT TAC GC	*phoQ*	
89-*phoQ*-Rev	GAT ATT CCA CTG CAG GTG TC	*phoQ*	
1011-*lpxM*-Fwd1	GGT TAA GAT TTT GCC TGG	*lpxM*	
1011-*lpxM*-Rev1	CTG GGG CAC CGG CTT TCC ACT GAC CGG TGG TAA GTG AGT GTG ATG TGA TAT TAT	*lpxM*	
1011-*lpxM*-Fwd2	CCA CCG GTC AGT GGA AAG	*lpxM*	
1011-*lpxM*-Rev2	TCC TCA CCG TCG AGT AAG	*lpxM*	

Chapter

5

In vitro evolution of colistin resistance in the *Klebsiella pneumoniae* complex follows multiple evolutionary trajectories with variable effects on fitness and virulence characteristics

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Manuscript submitted

Abstract

The increasing prevalence of multidrug-resistant Gram-negative opportunistic pathogens, including *Klebsiella pneumoniae*, has led to a resurgence in use of colistin as last-resort drug. Colistin is a cationic lipopeptide antibiotic that selectively acts on Gram-negative bacteria through electrostatic interactions with anionic phosphate groups of the lipid A moiety of lipopolysaccharides (LPS). Colistin resistance is mediated through modification of these phosphate groups with cationic groups (e.g. 4-amino-4-deoxy-L-arabinose (L-Ara4N), or phosphoethanolamine). Here, we study the evolutionary trajectories *in vitro* towards colistin resistance in nosocomial *K. pneumoniae* complex strains (three *K. pneumoniae sensu stricto* strains and one *K. variicola* subsp. *variicola* strain) and their impact on fitness and virulence characteristics.

Through population sequencing of cultures grown during an *in vitro* evolution experiment, under increasing colistin concentrations, we found that resistance develops through a combination of single nucleotide polymorphisms (SNPs), insertion and deletions (indels), and the integration of insertion sequence (IS) elements, affecting genes associated with LPS biosynthesis and modification, and capsule structures. The development of colistin resistance negatively impacted maximum growth rate of one *K. pneumoniae sensu stricto* strain. Colistin-resistant strains had lipid A modified through hydroxylation, palmitoylation, and L-Ara4N addition. Colistin-resistant *K. pneumoniae sensu stricto* strains exhibited cross-resistance to LL-37, contrasting with the *K. variicola* subsp. *variicola* strain. The susceptibility to human serum increased in two colistin-resistant strains. Virulence, as determined in a *Caenorhabditis elegans* survival assay, was higher in two colistin-resistant strains.

Our study suggests that nosocomial *K. pneumoniae* complex strains can rapidly develop colistin resistance through diverse evolutionary trajectories. We show that, in addition to SNPs and indels, screening for integration of IS elements in novel locations outside of *mgrB*, can possibly elucidate resistance mechanisms in colistin-resistant *K. pneumoniae*. These findings suggest that resistance in *K. pneumoniae* complex strains can rapidly emerge *de novo* upon exposure to colistin, effectively shortening the lifespan of this last-resort antibiotic for the treatment of infections with multidrug-resistant *Klebsiella*.

Author summary

Bacteria that frequently cause infections in hospitalised patients, are becoming increasingly resistant to antibiotics. Colistin is a positively charged antibiotic that is used for the treatment of infections with multidrug-resistant Gram-negative bacteria. Colistin acts by specifically interacting with the negatively charged LPS molecule in the outer membrane of Gram-negative bacteria. Colistin resistance is mostly mediated through modification of LPS to reduce its negative charge. Here, we use a laboratory evolution experiment to show that strains belonging to the *Klebsiella pneumoniae* complex, a common cause of multidrug-resistant hospital-acquired infections, can rapidly accumulate mutations that reduce the negative charge of LPS without an appreciable loss of fitness. Colistin resistance can lead to cross-resistance to an antimicrobial peptide of the human innate immune system and can increase virulence in a nematode model. These findings show that extensively resistant *K. pneumoniae* complex strains may rapidly develop resistance to the last-resort colistin via different evolutionary trajectories, while potentially retaining their ability to cause infections.

Introduction

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen and a leading cause of hospital-associated infections such as pneumonia, soft tissue infections, and urinary tract infections. *K. pneumoniae* may also asymptomatically colonize the skin, upper respiratory tract, and digestive tract of healthy individuals (1, 2). The *K. pneumoniae* complex is genetically diverse, with different phylogroups within the complex corresponding to different species and subspecies, each occupying specific niches (1, 2). The *K. pneumoniae sensu stricto* and *K. quasipneumoniae* phylogroups are associated with intestinal carriage, whilst the *K. variicola* phylogroup is associated with plants and bovine carriage (1, 3). Of all strains isolated from human infections and typed as *K. pneumoniae*, the majority is *K. pneumoniae sensu stricto*, but *K. variicola* and *K. quasipneumoniae* have also been found to cause infections in patients and are frequently misidentified as *K. pneumoniae* (4, 5). Although infections with strains from the *K. variicola* phylogroup are relatively rare, they have been associated with the highest mortality rate within the *K. pneumoniae* complex (3).

In recent years, *K. pneumoniae* complex strains have rapidly emerged as multidrug-resistant pathogens through acquisition of resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides, and have increasingly become resistant to carbapenems through the acquisition of carbapenemases (6–9). The increasing prevalence of multidrug resistance within the *K. pneumoniae* complex, and the lack of development of novel antibiotic classes effective against Gram-negative bacteria, have limited the available therapeutic options against multidrug-resistant *K. pneumoniae* complex strains. These limitations have prompted the resurgence in the use of the antibiotic colistin in treatment of infections by *K. pneumoniae* complex strains (10–13). After its introduction into clinical practice in the 1950s, colistin fell into disuse in human medicine in the 1970s because of the neuro- and nephrotoxic side effects associated with its use, and the development of safer classes of antibiotics. Due to the emergence multidrug-resistant Gram-negative opportunistic pathogens, like *K. pneumoniae*, it has recently regained clinical relevance as a last-line antibiotic (13–16).

Colistin (polymyxin E) is a cationic, amphipathic molecule composed of a fatty acid chain linked to a non-ribosomally synthesized decapeptide (17, 18). The mechanism of action of colistin relies on the selective presence of the negatively charged lipopolysaccharides (LPS) in the membranes of Gram-negative bacteria. The negative charges of LPS are carried by the anionic phosphate groups of the lipid A moiety of LPS, which enable colistin to bind through electrostatic

interactions (17–20). Insertion of colistin into the outer membrane leads to membrane permeabilization. The subsequent destabilization of the cytoplasmic membrane, where LPS is present after synthesis in the cytoplasm, and where it awaits transport to the outer membrane, ultimately leads to cell death (17, 19, 21, 22).

The increased use of colistin to treat infections with multidrug-resistant Gram-negative bacteria, especially in low- and middle-income countries (13), and the use of colistin in livestock farming, either therapeutically to treat enteric infections or as a growth promoter (23, 24), has led to a well-documented rise in colistin resistance in *K. pneumoniae* from clinical, veterinary, and environmental sources (9, 25–29). Colistin resistance in *K. pneumoniae* complex strains is mostly mediated through decoration of lipid A with cationic groups, to counteract the electrostatic interactions between colistin and lipid A (17). These modifications can be the result of point mutations and indels in chromosomally located genes (including *phoPQ*, *pmrAB*, and *crrAB*) resulting in amino acid substitutions and frameshift mutations, respectively. In addition, the acquisition of mobile genetic elements carrying a member of the *mcr*-gene family may also lead to lipid A modification (30–35). In *K. pneumoniae*, the inactivation of *mgrB* encoding an negative regulator of PhoPQ, through the insertion of an insertion sequence (IS) element, or a mutation leading to the formation of a premature stop codon, is a particularly frequently observed colistin resistance mechanism (25, 36–40). Other mechanisms of colistin resistance in *K. pneumoniae* include the upregulated expression of efflux pumps (41, 42), changes in LPS production (25, 43), and the overproduction of capsular polysaccharides (44).

Upon infection the innate immune system will attempt to neutralize invading bacteria, often in concert with antibiotic therapy. The cellular components of the innate immune system recognize microorganism-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) as a signal for activation (45). Since LPS is both exclusive to Gram-negative bacteria, and essential for *Klebsiella*, it is a crucial MAMP for the recognition of this pathogen (45). The lipid A moiety of LPS is recognized by LPS-binding protein and CD14, and subsequent transferred to the TLR4-MD2 PRR complex (45, 46). Activated phagocytes will then engulf the invading bacteria and will attempt to kill them by fusing phagosomes with lysosomes. Lysosomes contain numerous antimicrobial compounds, including the antimicrobial peptide LL-37. Similar to colistin, LL-37 relies on electrostatic interactions with LPS for its mechanism of action (47). In parallel to the cellular components of the innate immune system, the humoral immune system, including the complement system, also plays a vital role in the eradication of invading bacteria. The membranes, and other surface exposed

molecules, of invading bacteria play an important role in the initiation of the complement cascade (48). The complement system lyses bacteria through the membrane attack complex, or opsonises them for recognition by phagocytes (48). Modifications to LPS may influence these processes, and may thus result in altered virulence by reducing the activation and effectiveness of the immune system (45, 47–50). Modifications capable of affecting the efficiency of the immune system include neutralization of the anionic charges carried by lipid A, and changes in acylation of lipid A (45, 49–51). These changes are mediated through the PhoPQ and PmrAB two-component regulatory systems. Notably, colistin resistance is mediated through the same modifications and two-component regulatory systems. The development of colistin resistance may thus also affect virulence characteristics, and vice versa.

To better understand the mechanisms and consequences of colistin resistance in *K. pneumoniae* complex strains, we determined the evolutionary trajectories of three *K. pneumoniae sensu stricto* strains and one *K. variicola* subsp. *variicola* strain towards colistin resistance in an *in vitro* evolution experiment, and determined how colistin resistance impacted fitness, LPS modifications, and virulence characteristics.

Results

The colistin-susceptible *K. pneumoniae* complex strains have a diverse genetic background

The four randomly selected, nosocomial isolates used in this study were obtained from pus, faecal, or urine samples through routine diagnostic procedures in September 2013. All four strains were initially typed as *K. pneumoniae sensu stricto* through routine diagnostic procedures using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF). The susceptibility to colistin of these strains, previously determined in routine diagnostic procedures, was confirmed through antibiotic susceptibility testing using broth microdilution (Figure 1A).

The sequenced genomes of the colistin-susceptible strains were screened for acquired antibiotic resistance genes through ResFinder 3.1 (Figure 1B). None of the nosocomial strains was determined to carry one of the *mcr* genes. Between two and five acquired antibiotic resistance genes were observed in the genome assemblies, encoding resistance against ß-lactams, quinolones, and fosfomycin.

To accurately identify the phylogenetic position of these nosocomial strains within the *K. pneumoniae* complex, a phylogenetic tree was generated based on the Illumina/Oxford Nanopore hybrid genome assemblies of the colistin-susceptible strains, and 37 publicly available genomes covering all phylogroups in the *K. pneumoniae* complex (2). Based on a 1.3 Mbp core-genome alignment, the phylogenetic tree showed that strains KP209, KP040, and KP257 clustered in the *K. pneumoniae sensu stricto* (KpI) phylogroup (Figure 1C). Strain KV402 clustered in the *K. variicola* subsp. *variicola* (KpIII) phylogroup, even though it had been typed as *K. pneumoniae sensu stricto* through MALDI-TOF during initial routine diagnostic procedures.

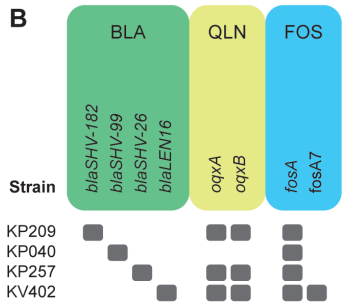
Colistin resistance emerges through multiple evolutionary trajectories in the *K. pneumoniae* complex

In an effort to understand the evolutionary trajectories through which the *K. pneumoniae* complex strains evolved resistance towards colistin, we deep-sequenced each overnight culture of the *in vitro* evolution experiment (Supplemental Table S1), in which the strains were grown in the presence of increasing concentrations of colistin and identified single nucleotide

A

Strain	Date of isolation	Isolation source	MLST type	MIC of colistin (mg/L)	Reference
KP209	17-09-2013	Urine	11	0.5	This study
KP040	09-09-2013	Faeces	10	1	This study
KP257	27-09-2013	Pus	3030	1	This study
KV402	19-09-2013	Urine	NA	0.5	This study

B



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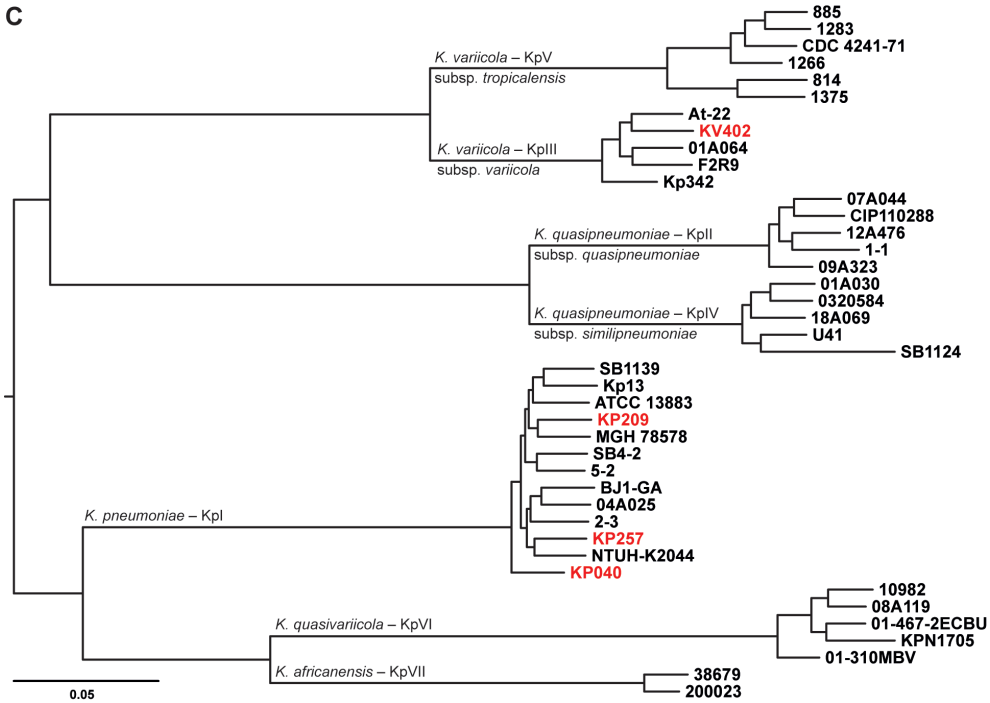

Figure 1: *K. pneumoniae* complex strains: metadata, presence of antibiotic resistance genes, and core-genome phylogenetic analysis. A) Overview of the isolates used in this study, including the date and source of isolation, MLST type, and the initial MIC determined. MLST typing of strain KV402 resulted in an incomplete MLST profile, so no conclusive ST could be assigned. NA, not applicable. **B)** Antibiotic resistance genes detected in *K. pneumoniae* complex strains sequenced as part of this study. Classes of antibiotic resistance genes are indicated as follow: BLA, ß-lactam resistance genes; QLN, quinolone resistance genes; FOS, fosfomycin resistance genes. The strains did not carry acquired colistin resistance genes of the *mcr* family. C) Midpoint rooted phylogenetic tree representing the 1.3 Mbp core genome alignment of 41 *K. pneumoniae* complex. Taxonomic phylogroups of the *K. pneumoniae* complex (2) are indicated along the branches. The strains used in this study are highlighted in red.

polymorphisms (SNPs), insertion and deletions (indels), and excision/integration events of IS elements.

Through these methods, we observed the rapid emergence and fixation of several mutations (Figure 2) in the presence of colistin. In three strains (KP209, KP257, and KV402), these mutations occurred in the genes encoding the PhoPQ two-component regulatory system after one day of culturing (Supplemental Table S2). The PhoPQ two-component regulatory system is a well-known mediator of colistin resistance in *K. pneumoniae* complex strains (30, 52). The G385S substitution identified in PhoQ of KP257 has been previously linked to colistin resistance (53), and the other mutations in *phoPQ* presumably confer colistin resistance to these strains as well. In strain KP040, we observed the integration of an IS*5* element (Supplemental Table S3, Supplemental Figure S7) in the promoter region of both the *crrAB* operon, which encodes a two-component regulatory system, that has previously been linked to colistin resistance (31), and the CrrAB-controlled *crrC* gene, which encodes an activator of the PmrAB two-component regulatory system (54). In addition, an intergenic SNP (located in promoter regions of *ecpR* or *phnC*) in strain KP040 became fixed in the population on the first day of culturing. Both EcpR and PhnC have not previously been associated with colistin resistance. Although other mutations, in other locations, also occurred during the first day of culturing, these mutations failed to become fixed in the population, and were either lost on subsequent days, or did not change in abundance over time.

On subsequent days of the *in vitro* evolution experiment, novel mutations in the populations were associated with additional increases in minimal inhibitory concentration (MIC) of colistin. New SNPs that were fixed in the populations were observed in *phoQ* (strain KP209 (day 5), and KV402 (day 6)), and *pmrB* (KP209 (day4)). In strain KP257, a SNP in *lptD* was first observed on day 3, and was then fixed in the population. The *lptD* gene encodes a barrel-shaped transporter that transports LPS onto the outer leaflet of the outer membrane. Mutations in genes located in the K-locus, involved in capsule synthesis, were also detected. In strain KV402 a 13 bp deletion was observed in *wcaJ* from day 3 onwards, leading to a premature stop-codon. In KP040 a new insertion of IS*102*, inactivating *wzc* was observed from day 4. In addition, a 12 bp insertion in the gene encoding the Rho transcription termination factor was observed in KP040. We did not observe any mutations in the *mgrB* gene, encoding the negative regulator of the PhoPQ two-component regulatory system, in these *in vitro* evolution experiments.

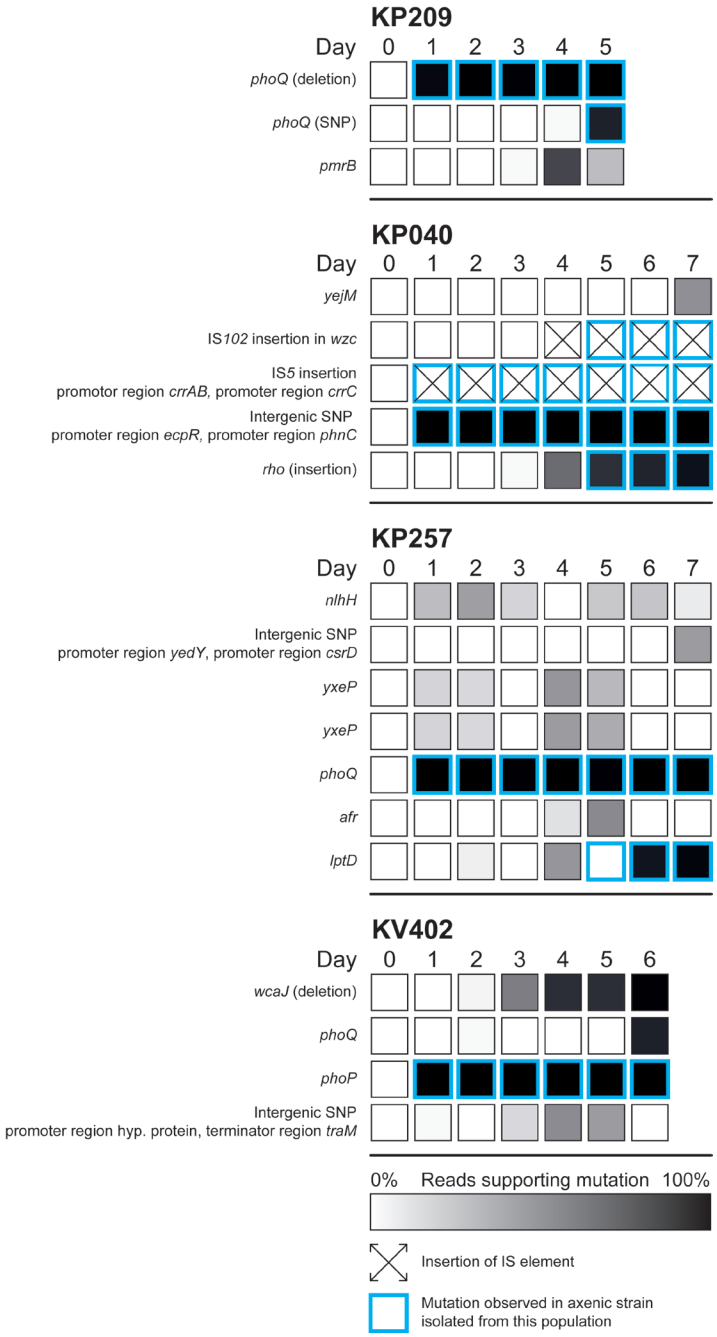

Figure 2: Population analysis of mutations during *in vitro* evolution in the presence of colistin. For each strain, and each day of the *in vitro* evolution experiment, the positions that have mutated compared to the colistin-susceptible strain are indicated. For SNPs and indels, the number of reads supporting a mutation at a given location was used to estimate the abundance of the mutation. Novel integrations of IS elements are also indicated. Mutations and IS element integrations observed in the axenic, strain isolated daily from each population are indicated by a blue border. For mutations not located in a coding sequence, nearby coding sequences are indicated. Hyp. protein: hypothetical protein.

K. pneumoniae can rapidly develop colistin resistance without loss of fitness.

To characterize changes in fitness and virulence characteristics as a result of development of colistin resistance, we isolated an axenic strain on each day of the *in vitro* evolution experiments. The genome sequences of the axenic strains of the last day of the *in vitro* evolution experiments were determined by Illumina sequencing. SNPs, indels and IS element insertions were identified in these strains in comparison with the colistin-susceptible parental strain. After combining these data with the population sequencing data described above, we determined the presence of these mutations in the axenic strains isolated after each day of the *in vitro* evolution experiment by targeted PCRs and Sanger sequencing of the amplicons. We were thus able to correlate the occurrence of mutations with increases in the MIC of colistin in each strain.

All four strains developed levels of resistance to colistin above the breakpoint value after one overnight incubation of the colistin-susceptible strain in the presence of the antibiotic (Figure 3). The initial mutations in *phoPQ* were associated with an increase in MIC in strains KP209, KP257, and KV402 (Figure 2; Figure 3). The integration of the IS*5* element in the promoter region of *crrAB* and *crrC*, and the appearance of an intergenic SNP between *ecpR* and *phnC*, also occur simultaneously with an increase in the MIC of colistin. The additional SNP in *phoQ* in KP209 was not associated with an increase in the MIC of colistin. Integration of IS*102* in *wzc* of the K-locus, as well as the 12 bp insertion in the gene encoding the transcription termination factor Rho, was associated with an additional increase

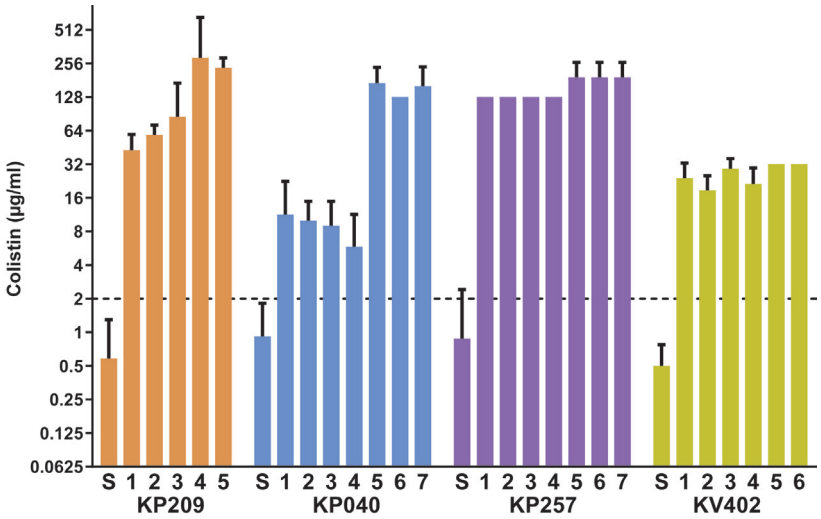

Figure 3: Development of colistin resistance in _K. pneumoniae_. MIC of colistin of axenic strains isolated from the *in vitro* evolution experiment. Colistin resistance was defined as an MIC > 2 µg/ml, according to EUCAST standards.

in the MIC of colistin in strain KP040. The SNP in *lptD* in strain KP257 did not lead to a meaningful increase in the MIC of colistin. The culture isolated from the last day of the KV402 *in vitro* evolution experiments had a SNP in *yciM* (Supplemental Table S4), encoding a negative regulator of LPS biosynthesis, but this did not contribute to a further reduced susceptibility to colistin.

The measurement of the maximum growth rate as a proxy for general fitness of the axenic strains isolated on the different days of the *in vitro* evolution experiment showed that the increase in MIC of colistin to values above 2 µg/ml after one overnight incubation, did not negatively affect the maximum growth rate for strains KP209, KP040, and KV402. Only the initial increase in MIC of colistin in strain KP257 had a negative impact on the maximum growth rate, decreasing the maximum growth rate by 37% (Figure 4). Over time, the maximum growth rates of strains KP209 and KV402 decreased 13.4% and 9.5%, respectively, compared to the maximum growth rate of the colistin-susceptible strain. In strain KP040, an increase of 10.0% in maximum growth rate was observed during the course of the *in vitro* evolution experiment.

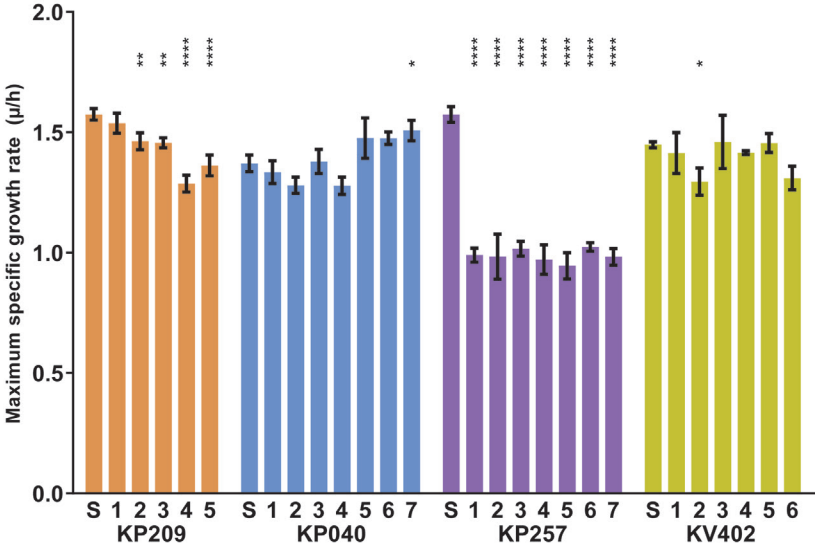

Figure 4: Maximum growth rate of colistin-resistant evolved strains. Optical density at 600 nm (OD600) was measured every 7.5 minutes. Representative data of three individual experiments, performed in triplicate are shown. Mean and standard deviations are shown. A parametric one-way ANOVA with Dunnett's multiple correction was used for the statistical analysis of the differences in growth rates between the axenic strains isolated from each day of the *in vitro* evolution experiment and the colistin-susceptible parental strain. Outcomes of the statistical analysis are indicated by asterisks: $p < 0.05$ (*), < 0.01 (**), <0.001 (***), or <0.0001 (****).

Colistin-resistant *K. pneumoniae* complex strains have lipid A that is modified through hydroxylation, palmitoylation and addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N)

The MALDI-TOF spectra of lipid A isolated from colistin-susceptible strains (Figure 5A), showed a dominant peak from hexa-acylated lipid A (mass-to-charge ratio (*m/z*) 1824), corresponding to two glucosamines, two phosphates, four 3-OH-C_{14} and two C_{14} acyl chains (55). Additional minor peaks in the MALDI-TOF spectrum of the susceptible strains could be observed at *m/z* 1840, corresponding to the hydroxylation (*m/z* 16) of one of the C_{14} acyl-groups of hexa-acylated lipid A (*m/z* 1824), and at *m/z* 2063 (in KP209 and KP257), corresponding to a hepta-acylated lipid A, with an additional acylation of lipid A (*m/z* 1824) with a palmitoyl group (*m/z* 239).

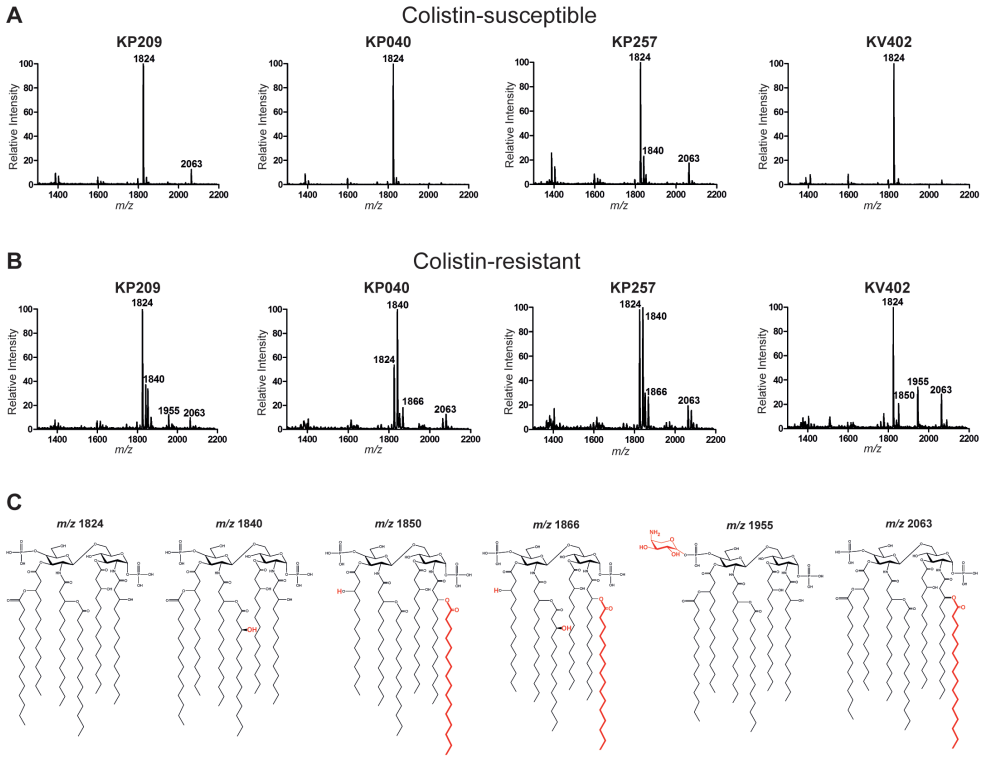

Figure 5: Lipid A modifications in colistin-susceptible and colistin-resistant strains. MALDI-TOF spectra showing the mass to charge (*m/z*) ratio values of the isolated lipid A from **(A)** colistin-susceptible, and **(B)** colistin-resistant axenic strains, isolated from the cultures of the last day of the *in vitro* evolution experiment. **C)** Proposed chemical structures of lipid A moieties corresponding to the observed *m/z* values in the MALDI-TOF spectra. Modifications relative to the unmodified hexa-acylated lipid A corresponding to *m/z* value 1824 are depicted in red. Hydroxylation of an acyl-chain adds 16 to the *m/z* ratio, 4-amino-4-deoxy-L-arabinose adds 131, acylation with palmitate adds 239.

All the MALDI-TOF spectra of lipid A isolated from colistin-resistant strains show additional peaks (Figure 5B), indicating the modification of their lipid A. In the spectra of colistin-resistant KP209 and KV402, lipid A *m/z* 1955 was observed, indicating addition of L-Ara4N (*m/z* 131) to the hexa-acylated lipid A *m/z* 1824. In colistin-resistant KV402 lipid A *m/z* 1850 was observed, consistent with hexa-acylated lipid A *m/z* 1824 with one C_{16} acyl chain (Figure 5C). The peak at *m/z* 1866 in the MALDI-TOF spectra of colistin-resistant KP040 and KP257 was consistent with hydroxylation of lipid A *m/z* 1850.

Development of colistin resistance is associated with increased LL-37 resistance, a decrease in serum susceptibility, and altered virulence in a *C. elegans* survival model.

Since the mechanisms used for immune evasion through LPS modification, and colistin resistance are similar, we investigated the effects of colistin resistance on virulence characteristics. We observed that three of the four colistin-resistant strains (KP209, KP040, and KP257) showed a decreased susceptibility to killing by LL-37 compared to their colistin-susceptible parental strains (Figure 6). In contrast, development of colistin resistance in strain KV402 did not affect susceptibility to LL-37.

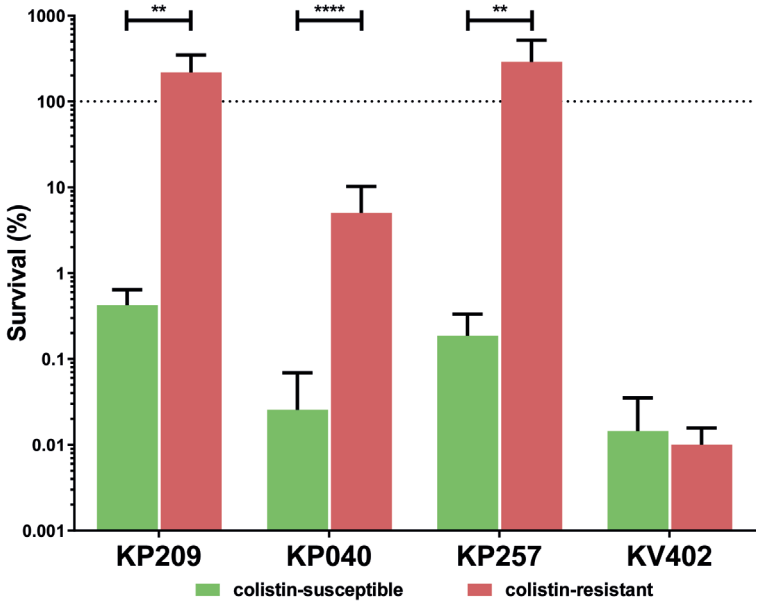

Figure 6: Susceptibility of colistin-susceptible and colistin-resistant strains to the human cathelicidin LL-37. Strains were incubated for 90 minutes in 25% LB at 37°C with or without the addition of 50 µg/ml LL-37. Viability was assessed by determination of the number of colony-forming units. The non-parametric Mann-Whitney test was used as statistical test and significance was defined as a p-value of < 0.05 (*), < 0.01 (**), <0.001 (***), or <0.0001 (****).

When exposed to the humoral immune system in human serum, we observed an increased susceptibility in two of four colistin-resistant strains. In 50% human serum, the colistin-resistant KP257 strain exhibited a decreased survival compared to its colistin-susceptible parental strain (Figure 7A). There was no difference in susceptibility of 50% serum in colistin-susceptible and -resistant variants of strains KP209 and KV402. Strain KP040 was notably resistant to 50% serum. In 10% human serum, a difference in survival between the colistin-susceptible and colistin-resistant KP209 was observed (Figure 7B). The colistin-susceptible and -resistant KP257 strains were unaffected by 10% serum. No differences between colistin-susceptible and colistin-resistant KV402 were observed. All bactericidal effects of serum were abolished when the serum was heat inactivated, indicating that the observed differences in serum-survival were attributable to heat-labile

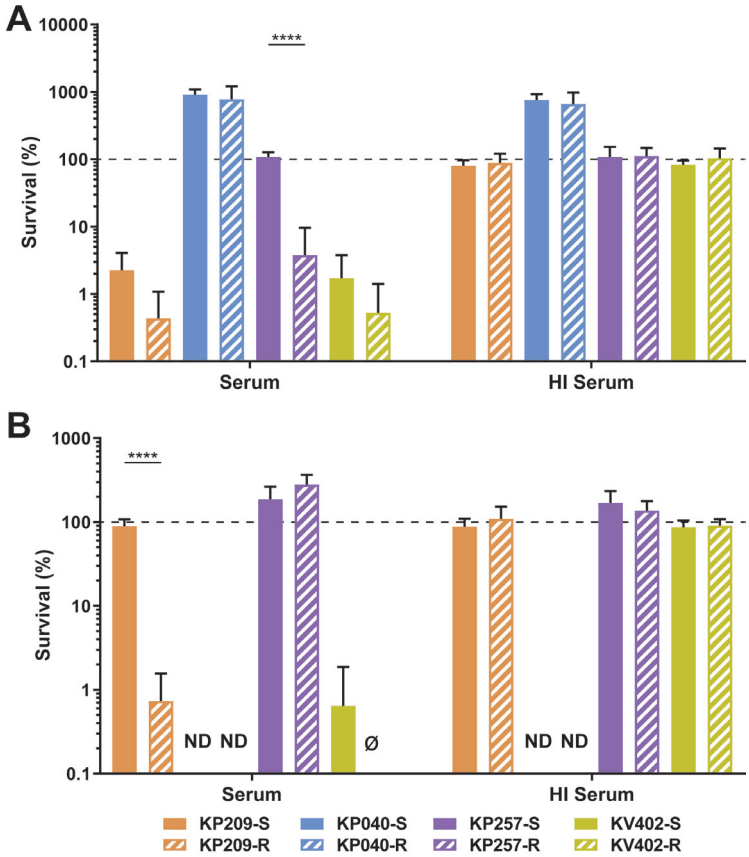

Figure 7: Survival of colistin-susceptible and colistin-resistant strains in human serum. Survival of *K. pneumoniae* complex colistin-susceptible and colistin-resistant strains after 1 hour incubation in **A)** 50% serum, or **B)** 10% serum. Heat-inactivated serum (HI) was used as a control. ND, not determined. Ø, not detected. Bars are coloured per isolate, and patterned according to status of resistance (-S; colistin-susceptible, -R; colistin-resistant). Statistical tests were performed using a one way ANOVA test, with Sidak's multiple comparisons test, and significance was defined as a p-value of < 0.05 (*), < 0.01 (**), <0.001 (***), or <0.0001 (****).

components.

To investigate the possible consequences of colistin resistance on *in vivo* virulence, we exposed the nematode *Caenorhabditis elegans* strain CF512 to the colistin-susceptible/resistant strain pairs. *C. elegans* had a decreased lifespan on a lawn of colistin-resistant KP209 (Figure 8) and KP040, compared to their colistin-susceptible strains. Survival of *C. elegans* was not affected by growth on colistin-resistant strains derived from KP257 and KV402, compared to the colistin-susceptible parental strains.

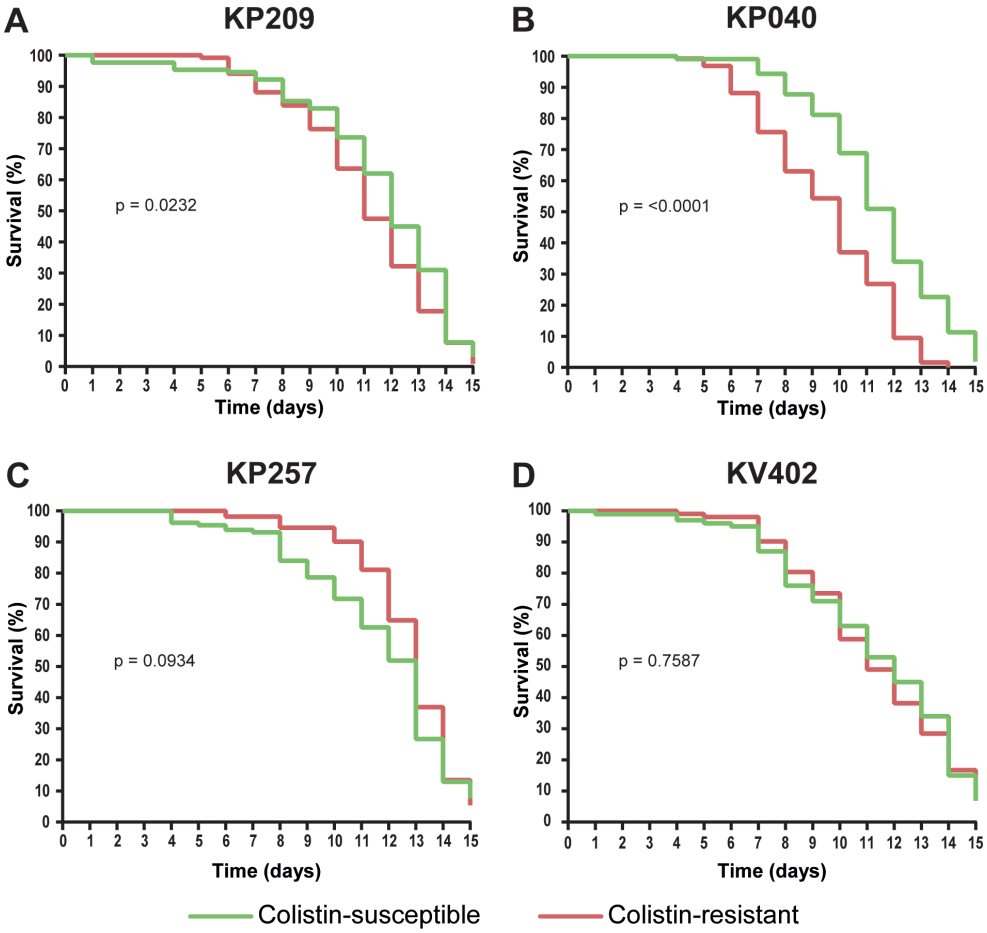

Figure 8: Survival of *C. elegans* on lawns of *K. pneumoniae* colistin-susceptible and colistin-resistant strains complex strains. C. elegans CF512 were kept on a lawn of colistin-susceptible (green) and colistin-resistant (red) *K. pneumoniae* complex strains. Survival was scored over a period of 15 days. The data represent three independent experiments in which a total of 129 (in colistin-susceptible KP209), 118 (colistin-resistant KP209), 106 (colistin-suscptible KP040), 127 (colistin-resistant KP040), 127 (colistin-susceptible KP257), 131 (colistin-resistant KP257), 100 (colistin-susceptible KP402), and 102 (colistin-resistant KP402) *C. elegans* nematodes were used. Statistical significance according to Mantel-Cox log-rank test is indicated. Statistical significance was defined as a p-value < 0.05.

Discussion

Colistin plays a pivotal role in public health due to its last-resort status for treatment of infections with multidrug-resistant Gram-negative bacteria. The increasing number of reports of *K. pneumoniae* strains that have acquired resistance to multiple antibiotics, including colistin, is thus a cause for increasing concern (7–9, 29). In this study, we observed the swift development of colistin resistance through diverse evolutionary trajectories by conducting an *in vitro* evolution experiment with nosocomial *K. pneumoniae* complex strains. Development of colistin resistance had no, or only a minor, impact on fitness in three out of four *in vitro* evolution experiments performed here, suggesting that colistin may rapidly lose its effectiveness in the treatment of infections caused by multidrug-resistant *K. pneumoniae* complex strains.

We observe that mutations associated with an increase in MIC of colistin seem confined to genes from functional groups involved in the synthesis and modification of LPS, and the synthesis of capsular polysaccharides, which are both important surface-associated structures. In the genes encoding the PhoPQ two-component regulatory system, which have a role in modification of LPS and which have been extensively described to facilitate colistin resistance in Enterobacteriaceae (30, 34, 56), we find variations in both PhoP (a D191N substitution), and PhoQ (a G385S substitution, and a 12 bp deletion). The G385S PhoQ substitution has previously been described in a clinical strain of colistin-resistant *K. pneumoniae* (53). Outside PhoPQ, we found that a novel integration of an IS*5* element in the promoter region associated with the genes encoding CrrAB and CrrC coincides with increase in MIC of colistin. The IS*5* element can influence the transcriptional activity of the genes located near its integration site (57). The activity of PmrAB may be influenced by CrrAB through CrrC (31, 54, 58). In line with previous observations, in which the novel insertions of IS elements were associated with resistance to tigecycline and colistin, we hypothesize that the insertion of IS*5* may lead to increased expression of either CrrAB and/or CrrC, and cause colistin resistance (40, 59).

For the functional group involved in capsule synthesis, we observe that the inactivation of *wzc* of the K-locus, by the IS*102* element coincides with an increase in the MIC of colistin. Wzc has previously been hypothesised to be involved in colistin resistance in *E. coli*, and it may act similarly in *K. pneumoniae* (60–62). The loss of Wzc may potentially cause colistin resistance through two mechanisms. A reduction in the export of colanic acid units (the building blocks of *K. pneumoniae* capsule), will lead to the accumulation of colanic acid metabolic intermediates, including UDP-glucuronic acid. This accumulation has been hypothesised to

lead to an increased flux towards biosynthesis of UDP-L-Ara4N, resulting in the modification of lipid A with L-Ara4N (63). Alternatively, the absence or reduction of negatively charged colanic acid residues on the cell surface could lower local concentrations of positively charged colistin molecules, thereby reducing damage to the outer membrane (63). Together with the inactivation of *wzc*, we observe a 12 bp insertion in the highly-conserved *rho* gene, encoding the transcription termination factor Rho. Rho has not been previously linked to colistin resistance, but mutations in *rho* may have pleiotropic effects on transcription (64), which could influence the expression of genes involved in, or may compensate for fitness costs caused by, colistin resistance.

Notably, we did not find any alterations in *mgrB*, which is an otherwise important mechanism through which colistin resistance may occur in nosocomial *K. pneumoniae* complex strains (25, 36–39). Nevertheless colistin-resistant clinical *K. pneumoniae* isolates without mutations in *mgrB* are also frequently encountered (53, 54, 65–68). We can only speculate on the reasons for the absence of *mgrB* mutations in our *in vitro* evolution experiments, but the relatively short duration of this experiment performed with a limited number of strains, could means that we have not covered all potential colistin resistance mechanisms in *K. pneumoniae*.

The impact of developing colistin resistance might extend past the inability to treat the infection through antibiotic therapy, as modifications to lipid A may reduce the susceptibility to antimicrobial peptides, or increase virulence, as we show in this study. The modifications to lipid A observed in this study may facilitate evasion from the cellular parts of the immune system (55, 69–71). We noted an increased susceptibility to the humoral immune system in two colistin-resistant strains, suggesting that at least some colistin-resistant strains may be quickly eradicated through the actions of serum-based defence mechanisms upon their entry in the bloodstream. However, the high serum resistance and increased virulence in a nematode model of infection of colistin-resistant KP040, suggests that colistin-resistance in *K. pneumoniae* can readily emerge in a strain that is resistant to serum-mediated killing without affecting fitness. However, the mechanisms behind the differential effects on virulence of colistin resistance in the *K. pneumoniae* complex are not fully understood.

Our data show that *K. pneumoniae* complex strains can rapidly develop resistance to colistin. Notably, a single *K. variicola* isolate was included in our study. As *K. variicola* can cause life-threatening infections in immunocompromised individuals (5), but is currently understudied, additional studies into the mechanisms of colistin resistance and their impact on fitness and virulence may be warranted in this species. To prevent the rapid emergence of colistin resistance in *K. pneumoniae* complex strains in clinical settings, the use of colistin in synergistic combinations

with other antibiotics may limit development of resistance (72). Additionally, colistin resistance in *K. pneumoniae* may confer collateral sensitivity to other classes of antibiotics, and may yield combinations of antibiotics that can be used alternately, in a process termed drug cycling (73, 74). Although a diverse set of two-component systems may be implicated in the development of resistance, the PmrAB two-component system appears to play a central role since both the PhoPQ and CrrAB two-component systems activate PmrAB, through PmrD and CrrC respectively (54). The development of a small-molecule inhibitor targeting the two-component systems involved in lipid A modifications, and in particular PmrAB, may be essential to lengthen the clinical lifespan of colistin as a last-resort drug in treatment of *K. pneumoniae* complex strains.

Materials and Methods

Ethical statement

The colistin-susceptible *K. pneumoniae* complex strains used in this study were isolated as part of routine diagnostic procedures, which did not require consent or ethical approval by an institutional review board.

Bacterial strains, growth conditions, and chemicals

The colistin-susceptible KP209, KP040, KP257, and KV402 strains were retrospectively, obtained from the diagnostic laboratory of the University Medical Center Utrecht in Utrecht, the Netherlands. In initial routine diagnostic procedures, they were identified as *K. pneumoniae sensu stricto* by MALDI-TOF on a Bruker microflex system (Leiderdorp, the Netherlands). Colistin susceptibility testing of the clinical isolates was initially performed on a BD Phoenix automated identification and susceptibility testing system (Becton Dickinson, Vianen, the Netherlands). All strains were grown either in lysogeny broth (LB; Oxoid, Landsmeer, the Netherlands) with agitation at 300 rpm, or on LB agar, at 37°C, unless otherwise specified. Colistin sulphate was obtained from Duchefa Biochemie (Haarlem, the Netherlands).

Determination of minimal inhibitory concentration of colistin

MICs of colistin were determined as described previously (75) in line with the recommendations from the joint Clinical & Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Polymyxin Breakpoints Working Group (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf). In short, colistin susceptibility testing was performed using BBL™ Mueller Hinton II (cation-adjusted) broth (MHCAB; Becton Dickinson), untreated Nunc 96-wells round bottom polystyrene plates (Thermo Fisher Scientific, Landsmeer, the Netherlands), and Breathe-Easy sealing membranes (Sigma-Aldrich, Zwijndrecht, the Netherlands). The MIC was observed after stationary, overnight growth at 37°C, and was determined to be the lowest concentration where no visible growth was observed. The breakpoint value for colistin resistance of an MIC > 2 µg/ml was obtained from EUCAST (76) (http://www.eucast.org/clinical_breakpoints/).

In vitro evolution of colistin resistance

The nosocomial, colistin-susceptible *K. pneumoniae* strains were evolved towards colistin resistance by culturing in increasing colistin concentrations over a period of 5-7 days. Prior to the *in vitro* evolution experiments, MICs of colistin were determined in LB. Each strain was grown in 1 ml LB with an initial colistin concentrations at 1 and 2 times the MIC. After overnight growth, 1 µl of the cultures with the highest concentration of colistin that had visible growth were used to propagate a fresh culture by inoculating 1 ml of fresh LB, supplemented with the same or twice the concentration of colistin in which growth was observed in the previous day's culture (Supplemental Table S5). This process was repeated for 5-7 days. Each overnight culture was stored at -80°C in 20% glycerol.

Genomic DNA isolation and whole-genome sequencing

Genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega, Leiden, the Netherlands) according to the manufacturer's instructions. DNA concentrations were measured with the Qubit 2.0 fluorometer and the Qubit dsDNA Broad Range Assay kit (Life Technologies, Bleiswijk, the Netherlands).

Illumina sequence libraries of genomic DNA were prepared using the Nextera XT kit (Illumina, San Diego, CA) according to the manufacturer's instructions, and sequenced on an Illumina MiSeq system with a 500-cycle (2 × 250 bp) MiSeq v2 reagent kit (Illumina). MinION library preparation for barcoded 2D long-read sequencing was performed using the SQK-LSK208 kit (Oxford Nanopore Technologies, Oxford, England, United Kingdom), according to the manufacturer's instructions, with G-tube (Covaris, Woburn, Massachusetts, United States of America) shearing for 2 x 120 seconds at 1500 x *g*. The libraries were sequenced on an MinION sequencer (Oxford Nanopore Technologies) through a SpotON Flow Cell Mk I (R9.4; Oxford Nanopore Technologies).

Genome assembly and annotation

The quality of the Illumina sequencing data was assessed using FastQC v0.11.5 (https://github.com/s-andrews/FastQC). Illumina sequencing reads were trimmed for quality using nesoni v0.115 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) using standard settings with the exception of a minimum read length of 100 nucleotides. MinION reads in FastQ format were extracted from Metrichor base-called FAST5-files using Poretools (77). *De novo* genome hybrid assembly of colistin-susceptible strains was performed

using Illumina and Oxford Nanopore data using SPAdes v3.6.2 with the following settings: kmers used: 21, 33, 55, 77, 99, 127, "careful" option turned on and cut-offs for final assemblies: minimum contig/scaffold size of 500 bp, and a minimum average scaffold nucleotide coverage of 10 (78, 79). Genome annotation was performed using Prokka (80).

Phylogenetic analysis, MLST typing, and identification of antibiotic resistance genes

To generate a core genome phylogeny, Illumina/Oxford Nanopore hybrid genome assemblies were aligned using Parsnp v1.2 (37) with 37 publicly available *K. pneumoniae* complex genomes that cover all phylogroups of the *K. pneumoniae* complex (2). To include the genome of *K. africanensis* strain 38679, we assembled the genome from raw reads, by processing the raw sequence reads using Nesoni with standard settings, except for minimum read length (75 nucleotides), and subsequent assembly by SPAdes with kmers 21, 33, 55, 77 and the "careful" options turned on.

Figtree was used to visualize and midpoint root the phylogenetic tree (http://tree.bio.ed.ac.uk/). MLST typing was performed using the mlst package v2.10 (https://github.com/tseemann/mlst). Genome assemblies of colistin-susceptible strains were assessed for antibiotic resistance genes by ResFinder 3.1 through standard settings (81).

Determination of SNPs and indels between axenic colistin-susceptible and colistin-resistant strain pairs.

Read-mapping of Nesoni-filtered reads of evolved strains to the genomes of the isogenic colistin-susceptible parental strains was performed using Bowtie2 (82). SNP and indel-calling was performed using SAMtools 0.1.18 using the following settings: Qscore ≥ 50, mapping quality ≥ 30, a mapping depth ≥ 10 reads, a consensus of ≥ 75% to support a call, and ≥ 1 read in each direction supporting a mutation, as previously described (83). To correct for potential assembly errors, we also performed the SNP and indel-calling procedure by mapping the reads of the reference isolates against their own assemblies. SNPs and indels found in the reference-versus-reference comparison were ignored in query-versus-reference comparisons. Synonymous mutations were excluded from further analyses. SNPs and indels were manually linked to genes in the assembly.

Determination of location of IS elements in genomes

To determine which IS elements were present in the genomes of colistin-susceptible strains, we analysed the Illumina/Oxford Nanopore hybrid genome assemblies using ISfinder (84). Per genome, the IS elements with an E-value < 1e-50 were selected for further study. If multiple distinct IS elements were called at the same position, the element with the highest sequence identity was selected to represent that position.

To detect changes in the position of the identified IS elements, we analysed the genomic assemblies of the isogenic colistin-susceptible and colistin-resistant strain pairs through ISMapper (85). To maximize the ability of ISMapper to detect IS elements in our sequencing data, the obtained nucleotide sequences of the IS elements in the genome were used as input, and the --cutoff flag of ISMapper was set to 1, whilst other settings remained unchanged. The results were inspected for IS elements that had different positions between the colistin-susceptible, and colistin-resistant strains. Insertion of IS elements was confirmed through PCRs, using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and primers spanning the IS insertion site (Supplemental Table S6) and subsequent Sanger sequencing of the PCR product by Macrogen (Amsterdam, the Netherlands).

SNP and indel calling in evolving populations.

To track the genomic changes within the growing cultures under the selective pressure of increasing colistin concentrations, genomic DNA was isolated from the 5-7 overnight cultures of each *in vitro* evolution experiment and sequenced on the Illumina MiSeq platform as described above. SNPs and indels were called as before, with each call supported by at least 25% of reads. Once identified in one or more populations, the abundance of the specific SNPs and indels were then quantified manually for all individual populations of the *in vitro* evolution experiment. Mutations called within 150 bp of a contig end were filtered out, as previously recommended (86). Identified SNPs and indels were manually linked to genes in the genome assembly, and inspected for synonymous versus non-synonymous mutations. Non-coding mutations were included in subsequent analyses, while synonymous mutations were excluded.

Determination of growth rate

To determine the maximum specific growth rate, a Bioscreen C instrument (Oy Growth Curves AB, Helsinki, Finland) was used. Overnight cultures were used

to inoculate 200 µl fresh LB medium 1:1000. Incubation was set at 37°C with continuous shaking. Growth was observed by measuring the absorbance at 600 nm every 7.5 minutes. Each experiment was performed in triplicate.

MALDI-TOF analysis of lipid A structures

Isolation of lipid A molecules and subsequent analysis by negative-ion MALDI-TOF mass spectrometry was performed as previously described (55, 70, 87). Briefly, *K. pneumoniae* strains were grown in LB (Oxoid) and the lipid A was purified from stationary cultures using the ammonium hydroxide/isobutyric acid isolation method described earlier (88). Mass spectrometry analysis were performed on a Bruker autoflex® speed TOF/TOF mass spectrometer in negative reflective mode with delayed extraction using as matrix an equal volume of dihydroxybenzoic acid matrix (Sigma-Aldrich) dissolved in (1:2) acetonitrile-0.1% trifluoroacetic acid. The ion-accelerating voltage was set at 20 kV. Each spectrum was an average of 300 shots. A peptide calibration standard (Bruker) was used to calibrate the MALDI-TOF. Further calibration for lipid A analysis was performed externally using lipid A extracted from *Escherichia coli* strain MG1655 grown in LB medium at 37°C.

LL-37 survival assay

In order to test the susceptibility of the *K. pneumoniae* strains to LL-37, we adapted previously described protocols (71). An overnight broth culture was diluted to a concentration of 2.5×10^6 CFU/ml in 25% LB and incubated with or without the addition of 50 µg/ml LL-37 (AnaSpec Inc, Fermont, California, United States of America) for 90 minutes at 37°C with agitation at 300 rpm in sterile round-bottom 96-well plates (Greiner Bio-One, Alphen aan den Rijn, the Netherlands). After incubation, samples were serially diluted in PBS and plated on LB agar plates. CFUs were counted after overnight incubation at 37°C.

Serum survival assay

Susceptibility to human pooled serum was determined as previously described (89). Bacteria were grown to mid-exponential phase from an overnight culture. Mid-exponential phase bacteria were diluted to 1×10^4 colony forming units (CFUs) per ml in RPMI supplemented with 0.05% human serum albumin (HSA) (Sanquin, Amsterdam, the Netherlands). RPMI (Gibco, Merelbeke, the Netherlands) diluted bacterial cultures were incubated with either 10% or 50% human pooled

serum, prepared as previously described (89), at 37°C with agitation at 300 rpm in round-bottom 96-wells plates (Greiner Bio-One, Alphen aan den Rijn, the Netherlands. Heat-inactivated serum was prepared by heating serum at 56°C for 30 minutes. After incubation, samples were serially diluted in phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl) and plated on LB agar plates. The number of colony-forming units (CFUs) were counted after overnight incubation at 37°C.

C. elegans virulence assays

C. elegans strain CF512 (*rrf-3*(*b26*) II; *fem-1*(*hc17*) IV), which has a temperature-sensitive reproduction defect, was obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota, Twin Cities (http://www.cgc.cbs.umn.edu/). CF512 nematodes were maintained at 20°C on Nematode Growth Medium (NGM) agar plates seeded with *E. coli* OP50 (90), and placed on fresh plates at least once per week. For seeding of NGM plates, mid-exponential phase cultures were used. After reaching mid-exponential phase, the cells were washed with PBS, and 1 x 10^6 CFU were spread on NGM plates, after which the bacterial lawns were grown overnight at 37°C.

C. elegans CF512 lifespan assays were performed with synchronized nematodes according to a previously described protocol (91). For synchronization, nematodes and eggs were collected from a NGM plate in ice-cold filter-sterilized M9 medium, and washed by spinning at 1500 x *g* for 30 seconds (92). Nematodes were destructed by vigorous vortexing in hypochlorite solution (25 mM NaOH, 1.28% sodium hypochlorite) for two minutes, after which the reaction was stopped by the addition of M9 medium. Eggs were allowed to hatch on NGM plates seeded with *E. coli* OP50 for 6-8 hours at 20°C, after which they were placed at 25°C to avoid progeny. After 48 hours, L3-L4 nematodes were placed on NGM plates (n=40 per plate) seeded with bacterial strains. Plates were scored for live nematodes. Nematodes were considered dead when they did not show spontaneous movement or a response to external stimuli.

Statistical analysis

Statistical analyses were performed using the parametric one-way ANOVA test with a Dunnett's test for multiple comparisons (for the determination of maximum growth rates), the non-parametric Mann-Whitney test was used (for the LL-37 survival assay), the parametric one-way ANOVA test with a Sidak's test for multiple

comparisons (for the serum survival assays), and the Mantel-Cox log-rank test (for the *C. elegans* assays). Statistical significance was defined as a p-value < 0.05 for all tests. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, California, United States of America).

Data availability

Sequence data of both the Illumina short-read, and the Oxford Nanopore long-read sequencing has been deposited in the European Nucleotide Archive (accession number PRJEB29521).

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References

1. Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, Jenney A, Connor TR, Hsu LY, Severin J, Brisse S, Cao H, Wilksch J, Gorrie C, Schultz MB, Edwards DJ, Nguyen K V, Nguyen TV, Dao TT, Mensink M, Minh VL, Nhu NTK, Schultsz C, Kuntaman K, Newton PN, Moore CE, Strugnell RA, Thomson NR. 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc Natl Acad Sci U S A 112:E3574–E3581.
2. Rodrigues C, Passet V, Rakotondrasoa A, Diallo TA, Criscuolo A, Brisse S. 2019. Description of *Klebsiella africanensis* sp. nov., *Klebsiella variicola* subsp. *tropicalensis* subsp. nov. and *Klebsiella variicola* subsp. *variicola* subsp. nov.. Res Microbiol 170:165–170.
3. Maatallah M, Vading M, Humaun Kabir M, Bakhrouf A, Kalin M, Nauclér P, Brisse S, Giske CG. 2014. *Klebsiella variicola* is a frequent cause of bloodstream infection in the Stockholm area, and associated with higher mortality compared to *K. pneumoniae*. PLoS One 9:e113539.
4. Mathers AJ, Crook D, Vaughan A, Barry KE, Vegesana K, Stoesser N, Parikh HI, Sebra R, Kotay S, Sarah Walker A, Sheppard AE. 2019. *Klebsiella quasipneumoniae* provides a window into carbapenemase gene transfer, plasmid rearrangements, and patient interactions with the hospital environment. Antimicrob Agents Chemother 63:1–12.
5. Rodríguez-Medina N, Barrios-Camacho H, Duran-Bedolla J, Garza-Ramos U. 2019. *Klebsiella variicola*: an emerging pathogen in humans. Emerg Microbes Infect 8:973–988.
6. World Health Organization. 2014. Antimicrobial resistance. Global report on surveillance.
7. European Centre for Disease Prevention and Control. 2019. Surveillance of antimicrobial resistance in Europe 2018.
8. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Eurosurveillance 19:20939.
9. Parisi SG, Bartolini A, Santacatterina E, Castellani E, Ghirardo R, Berto A, Franchin E, Menegotto N, De Canale E, Tommasini T, Rinaldi R, Basso M, Stefani S, Palù G. 2015. Prevalence of *Klebsiella pneumoniae* strains producing carbapenemases and increase of resistance to colistin in an Italian teaching hospital from January 2012 to December 2014. BMC Infect Dis 15:244.
10. World Health Organization. 2019. 2019 Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline.
11. Shore CK, Coukell A. 2016. Roadmap for antibiotic discovery. Nat Microbiol 1:16083.
12. Llaca-Díaz JM, Mendoza-Olazarán S, Camacho-Ortiz A, Flores S, Garza-González E. 2013. One-year surveillance of ESKAPE pathogens in an intensive care unit of Monterrey, Mexico. Chemotherapy 58:475–481.
13. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, Goossens H, Laxminarayan R. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci 115:E3463–E3470.
14. Ozkan G, Ulusoy S, Orem A, Alkanat M, Mungan S, Yulug E, Yucesan FB. 2013. How does colistin-induced nephropathy develop and can it be treated? Antimicrob Agents Chemother 57:3463–3469.
15. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. Clin Infect Dis 40:1333–1341.
16. Lim LM, Ly N, Anderson D, Yang JC, Macander L, Jarkowski A, Forrest A, Bulitta JB, Tsuji BT. 2010. Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. Pharmacotherapy 30:1279–1291.
17. Velkov T, Thompson PE, Nation RL, Li J. 2010. Structure-activity relationships of polymyxin antibiotics. J Med Chem 53:1898–1916.
18. Domingues MM, Inácio RG, Raimundo JM, Martins M, Castanho MARB, Santos NC. 2012. Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. Biopolymers 98:338–344.
19. Landman D, Georgescu C, Martin DA, Quale J. 2008. Polymyxins revisited. Clin Microbiol Rev 21:449–465.
20. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. 2011. Molecular mechanisms of antibiotic resistance. Chem Commun 47:4055–4061.
21. Putker F, Bos MP, Tommassen J. 2015. Transport of lipopolysaccharide to the Gram-negative bacterial cell surface. FEMS Microbiol Rev 39:985–1002.

22. Sabnis A, Klöckner A, Becce M, Evans LE, Furniss RCD, Mavridou DAI, Stevens MM, Edwards AM. 2018. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. bioRxiv 479618v2.
23. Walsh TR, Wu Y. 2016. China bans colistin as a feed additive for animals. Lancet Infect Dis 16:1102–1103.
24. Timmerman T, Dewulf J, Catry B, Feyen B, Opsomer G, Kruif A de, Maes D. 2006. Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium. Prev Vet Med 74:251–263.
25. Halaby T, Kucukkose E, Janssen AB, Rogers MRC, Doorduijn DJ, van der Zanden AGM, al Naiemi N, Vandenbroucke-Grauls CMJE, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. Antimicrob Agents Chemother 60:6837–6843.
26. Kieffer N, Aires-de-Sousa M, Nordmann P, Poirel L. 2017. High rate of MCR-1–producing *Escherichia coli* and *Klebsiella pneumoniae* among pigs, Portugal. Emerg Infect Dis 23:2023–2029.
27. Wang X, Liu Y, Qi X, Wang R, Jin L, Zhao M, Zhang Y, Wang Q, Chen H, Wang H. 2017. Molecular epidemiology of colistin-resistant Enterobacteriaceae in inpatient and avian isolates from China: high prevalence of *mcr*-negative *Klebsiella pneumoniae*. Int J Antimicrob Agents 50:536–541.
28. Tuo H, Yang Y, Tao X, Liu D, Li Y, Xie X, Li P, Gu J, Kong L, Xiang R, Lei C, Wang H, Zhang A. 2018. The prevalence of colistin resistant strains and antibiotic resistance gene profiles in Funan river, China. Front Microbiol 9:3094.
29. Elemam A, Rahimian J, Mandell W. 2009. Infection with panresistant *Klebsiella pneumoniae*: a report of 2 cases and a brief review of the literature. Clin Infect Dis 49:271–274.
30. Olaitan AO, Morand S, Rolain J-M. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643.
31. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adamsa MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. Antimicrob Agents Chemother 59:536–543.
32. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168.
33. Nang SC, Li J, Velkov T. 2019. The rise and spread of *mcr* plasmid-mediated polymyxin resistance. Crit Rev Microbiol 45:131–161.
34. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev 30:557–596.
35. Sun J, Zhang H, Liu YH, Feng Y. 2018. Towards understanding MCR-like colistin resistance. Trends Microbiol 26:794–808.
36. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, the COLGRIT Study Group, Vatopoulos A, Rossolini GM. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob Agents Chemother 58:5696–5703.
37. Jayol A, Poirel L, Villegas M-V, Nordmann P. 2015. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. Int J Antimicrob Agents 46:108–110.
38. Cannatelli A, Santos-Lopez A, Giani T, Gonzalez-Zorn B, Rossolini GM. 2015. Polymyxin resistance caused by *mgrB* inactivation is not associated with significant biological cost in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 59:2898–2900.
39. Aires CAM, Pereira PS, Asensi MD, Carvalho-Assef APD. 2016. MgrB mutations mediating polymyxin B resistance in *Klebsiella pneumoniae* isolates from rectal surveillance swabs in Brazil. Antimicrob Agents Chemother 60:6969–6972.
40. Yang T, Wang S, Lin J-E, Griffith BTS, Lian S, Hong Z, Lin L, Lu P, Tseng S. 2020. Contributions of insertion sequences conferring colistin resistance in *Klebsiella pneumoniae*. Int J Antimicrob Agents 53:105894.
41. Ni W, Li Y, Guan J, Zhao J, Cui J, Wang R, Liu Y. 2016. Effects of efflux pump inhibitors on colistin resistance in multidrug-resistant Gram-negative bacteria. Antimicrob Agents Chemother 60:3215–3218.
42. Padilla E, Llobet E, Doménech-Sánchez A, Martínez-Martínez L, Bengoechea JA, Albertí S. 2010. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob Agents Chemother 54:177–183.
43. Mahalakshmi S, Sunayana MR, Saisree L, Reddy M. 2014. *yciM* is an essential gene required for regulation of lipopolysaccharide synthesis in *Escherichia coli*. Mol Microbiol 91:145–157.

44. Llobet E, Tomás JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154:3877–3886.
45. Needham BD, Trent MS. 2013. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol 11:467–481.
46. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458:1191–1196.
47. Gruenheid S, Le Moual H. 2012. Resistance to antimicrobial peptides in Gram-negative bacteria. FEMS Microbiol Lett 330:81–89.
48. Doorduijn DJ, Rooijakkers SHM, van Schaik W, Bardoel BW. 2016. Complement resistance mechanisms of *Klebsiella pneumoniae*. Immunobiology 221:1102–1109.
49. Maeshima N, Fernandez RC. 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol 3:3.
50. Raetz CR, Reynolds MC, Trent SM, Bishop RE. 2007. Lipid A modification in Gram-negative bacteria. Annu Rev Biochem 76:295–329.
51. Matsuura M. 2013. Structural modifications of bacterial lipopolysaccharide that facilitate Gram-negative bacteria evasion of host innate immunity. Front Immunol 4:109.
52. Jeannot K, Bolard A, Plesiat P. 2017. Resistance to polymyxins in Gram-negative organisms. Int J Antimicrob Agents 49:526–535.
53. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Chaisiri K, Komalamisra C, Adelowo OO, Fagade OE, Banjo OA, Oke AJ, Adler A, Assous MV, Morand S, Raoult D, Rolain JM. 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. Int J Antimicrob Agents 44:500–507.
54. Cheng Y-H, Lin T-L, Lin Y-T, Wang J-T. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 60:3709–3716.
55. Llobet E, Martínez-Moliner V, Moranta D, Dahlström KM, Regueiro V, Tomás A, Cano V, Pérez-Gutiérrez C, Frank CG, Fernández-Carrasco H, Insua JL, Salminen TA, Garmendia J, Bengoechea JA. 2015. Deciphering tissue-induced *Klebsiella pneumoniae* lipid A structure. Proc Natl Acad Sci U S A 112:E6369–E6378.
56. Li J, Nation RL, Kaye KS. 2019. Polymyxin antibiotics: from laboratory bench to bedside.
57. Schnetz K, Rak B. 1992. IS*5*: a mobile enhancer of transcription in *Escherichia coli*. Proc Natl Acad Sci 89:1244–1248.
58. Aghapour Z, Gholizadeh P, Ganbarov K, Bialvaei AZ, Mahmood SS, Tanomand A, Yousefi M, Asgharzadeh M, Yousefi B, Samadi Kafil H. 2019. Molecular mechanisms related to colistin resistance in Enterobacteriaceae. Infect Drug Resist 12:965–975.
59. Nielsen LE, Snesrud EC, Onmus-Leone F, Kwak YI, Avilés R, Steele ED, Sutter DE, Waterman PE, Lesho EP. 2014. IS*5* element integration, a novel mechanism for rapid *in vivo* emergence of tigecycline nonsusceptibility in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 58:6151–6156.
60. Obadia B, Lacour S, Doublet P, Baubichon-Cortay H, Cozzone AJ, Grangeasse C. 2007. Influence of tyrosine-kinase Wzc activity on colanic acid production in *Escherichia coli* K12 cells. J Mol Biol 367:42–53.
61. Lacour S, Bechet E, Cozzone AJ, Mijakovic I, Grangeasse C. 2008. Tyrosine phosphorylation of the UDP-glucose dehydrogenase of *Escherichia coli* is at the crossroads of colanic acid synthesis and polymyxin resistance. PLoS One 3:e3053.
62. Grangeasse C, Obadia B, Mijakovic I, Deutscher J, Cozzone AJ, Doublet P. 2003. Autophosphorylation of the *Escherichia coli* protein kinase Wzc regulates tyrosine phosphorylation of Ugd, a UDP-glucose dehydrogenase. J Biol Chem 278:39323–39329.
63. Pal S, Verma J, Mallick S, Rastogi SK, Kumar A, Ghosh AS. 2019. Absence of the glycosyltransferase WcaJ in *Klebsiella pneumoniae* ATCC13883 affects biofilm formation, increases polymyxin resistance and reduces murine macrophage activation. Microbiology 165:891–904.
64. Ciampi MS. 2006. Rho-dependent terminators and transcription termination. Microbiology 152:2515–2528.
65. Jayol A, Poirel L, Brink A, Villegas M-V, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. Antimicrob Agents Chemother 58:4762–4766.
66. Cheng Y-H, Lin T-L, Pan Y-J, Wang Y-P, Lin Y-T, Wang J-T. 2015. Colistin-resistant mechanisms of *Klebsiella pneumoniae* in Taiwan. Antimicrob Agents Chemother 59:2909–2913.

67. Choi MJ, Ko KS. 2014. Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. J Antimicrob Chemother 69:275–277.
68. Cheong HS, Kim SY, Wi YM, Peck KR, Ko KS. 2019. Colistin heteroresistance in *Klebsiella pneumoniae* isolates and diverse mutations of PmrAB and PhoPQ in resistant subpopulations. J Clin Med 8:1444.
69. Mills G, Dumigan A, Kidd T, Hobley L, Bengoechea JA. 2017. Identification and characterization of two *Klebsiella pneumoniae lpxL* lipid A late acylstransferases and their role in virulence. Infect Immun 85:e00068-17.
70. Kidd TJ, Mills G, Sá-Pessoa J, Dumigan A, Frank CG, Insua JL, Ingram R, Hobley L, Bengoechea JA. 2017. A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence. EMBO Mol Med 9:430–447.
71. Napier BA, Burd EM, Satola SW, Cagle SM, Ray SM, McGann P, Pohl J, Lesho EP, Weiss DS. 2013. Clinical use of colistin induces cross-resistance to host antimicrobials in *Acinetobacter baumannii*. mBio 4:e00021-13.
72. Brochado AR, Telzerow A, Bobonis J, Banzhaf M, Mateus A, Selkrig J, Huth E, Bassler S, Zamarreño Beas J, Zietek M, Ng N, Foerster S, Ezraty B, Py B, Barras F, Savitski MM, Bork P, Göttig S, Typas A. 2018. Species-specific activity of antibacterial drug combinations. Nature 559:259–263.
73. Imamovic L, Sommer MOA. 2013. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. Sci Transl Med 5:204ra132.
74. Imamovic L, Ellabaan MMH, Dantas Machado AM, Citterio L, Wulff T, Molin S, Krogh Johansen H, Sommer MOA. 2018. Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. Cell 172:121-134.e14.
75. Andrews JM. 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48:5–16.
76. Satlin MJ, Weinstein MP, Patel J, Romney M, Kahlmeter G, Giske CG, Turnidge J. 2020. Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) position statements on polymyxin B and colistin clinical breakpoints. Clin Infect Dis:ciaa121.
77. Loman NJ, Quinlan AR. 2014. Poretools: a toolkit for analyzing nanopore sequence data. Bioinformatics 30:3399–3401.
78. van Mansfeld R, de Been M, Paganelli F, Yang L, Bonten M, Willems R. 2016. Within-host evolution of the Dutch high-prevalent *Pseudomonas aeruginosa* clone ST406 during chronic colonization of a patient with cystic fibrosis. PLoS One 11:e0158106.
79. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin A V, Sirotkin A V, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477.
80. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.
81. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644.
82. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359.
83. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.
84. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 34:D32–D36.
85. Hawkey J, Hamidian M, Wick RR, Edwards DJ, Billman-Jacobe H, Hall RM, Holt KE. 2015. ISMapper: Identifying transposase insertion sites in bacterial genomes from short read sequence data. BMC Genomics 16:667.
86. Briskine R V., Shimizu KK. 2017. Positional bias in variant calls against draft reference assemblies. BMC Genomics 18:263.
87. Llobet E, Campos MA, Giménez P, Moranta D, Bengoechea JA. 2011. Analysis of the networks controlling the antimicrobial-peptide-dependent induction of *Klebsiella pneumoniae* virulence factors. Infect Immun 79:3718–3732.
88. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res 46:1773–1778.
89. Berends ETM, Dekkers JF, Nijland R, Kuipers A, Soppe JA, van Strijp JAG, Rooijakkers SHM. 2013. Distinct localization of the complement C5b-9 complex on Gram-positive bacteria. Cell Microbiol 15:1955–1968.
90. Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:95–104.

91. Maman M, Carvalhal Marques F, Volovik Y, Dubnikov T, Bejerano-Sagie M, Cohen E. 2013. A neuronal GPCR is critical for the induction of the heat shock response in the nematode *C. elegans*. J Neurosci 33:6102–6111.
92. Porta-de-la-Riva M, Fontrodona L, Villanueva A, Cerón J. 2012. Basic *Caenorhabditis elegans* methods: synchronization and observation. J Vis Exp 64:e4019.

Supplemental Materials

Supplemental Table S1: Summary of the read data from the sequencing runs, and genome assembly information.[a]

Strain	Sample	Type	Size of genome assembly	Number of scaffolds	Illumina reads mapped to reference	Average coverage of mapped Illumina reads
KP209	Colistin-susceptible	Axenic	5,4630,23 bp	1 chromosome, 5 plasmids	4,976,524	224.3
	Colistin-resistant	Axenic	NA	NA	4,310,150	192.7
	Day 1	Population	NA	NA	5,273,935	226.1
	Day 2	Population	NA	NA	3,498,310	148.9
	Day 3	Population	NA	NA	6,034,443	265.1
	Day 4	Population	NA	NA	8,381,010	360.5
	Day 5	Population	NA	NA	10,284,032	439.1
KP040	Colistin-susceptible	Axenic	5,970,459 bp	1 chromosome, 1 plasmid	4,645,736	190.5
	Colistin-resistant	Axenic	NA	NA	1,387,900	56.9
	Day 1	Population	NA	NA	2,389,602	96.2
	Day 2	Population	NA	NA	2,145,375	83.7
	Day 3	Population	NA	NA	3,207,128	125.2
	Day 4	Population	NA	NA	3,232,802	124
	Day 5	Population	NA	NA	2,556,849	96.3
	Day 6	Population	NA	NA	3,835,645	148.7
	Day 7	Population	NA	NA	2,807,670	105.6
KP257	Colistin-susceptible	Axenic	5,557,780 bp	1 chromosome, 3 plasmids	2,688,902	119.1
	Colistin-resistant	Axenic	NA	NA	2,073,063	91.1
	Day 1	Population	NA	NA	4,120,472	172.3
	Day 2	Population	NA	NA	3,878,524	161.7
	Day 3	Population	NA	NA	4,176,568	173.4
	Day 4	Population	NA	NA	4,669,134	194.3
	Day 5	Population	NA	NA	3,334,800	139.7

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	Day 6	Population	NA	NA	4,707,153	193.4
	Day 7	Population	NA	NA	3,884,435	160.5
KV402	Colistin-susceptible	Axenic	5,629,276 bp	1 chromosome, 2 plasmids	2,110,840	92.9
	Colistin-resistant	Axenic	NA	NA	1,453,643	63.9
	Day 1	Population	NA	NA	6,070,185	255.2
	Day 2	Population	NA	NA	4,501,469	186.2
	Day 3	Population	NA	NA	5,664,425	234.4
	Day 4	Population	NA	NA	6,186,379	256.1
	Day 5	Population	NA	NA	5,829,343	241.2
	Day 6	Population	NA	NA	6,218,915	254.1

[a] Per strain, the sequenced samples are indicated. The axenic colistin-resistant strain was picked from the last day of the *in vitro* evolution experiment. For the colistin-susceptible parental strain, the assembly statistics for the Illumina/Oxford Nanopore hybrid genome assembly are indicated. For all samples, the number of reads mapped to the hybrid assembly of the colistin-susceptible parental strain, and the average coverage are indicated. NA, not applicable

Supplemental Table S2: Mutations observed in the populations of the *in vitro* evolution experiment.[a]

Strain	Associated feature(s)	Location	Scaffold	Reference	Mutant	Coding change
KP209	*phoQ*	4,336,315	Chromosome	G GGC GCA GCG TGA	G	97-WAQRN -> 97-C
	phoQ	4,336,791	Chromosome	C	A	R256S
	pmrB	4,692,375	Chromosome	T	A	Y209N
KP040	*yejM*	304,945	Chromosome	G	C	H20Q
	Intergenic SNP: promoter region *ecpR*, promoter region *phnC*	2,954,735	Chromosome	T	G	*Not applicable*
	rho	3,907,646	Chromosome	C	C GCG ATG TTC TGC	189-QS -> 189-QNIAQS
KP257	*nlhH*	175,249	Chromosome	C	CCA	155-GGHLALVTALRLK… -> 155-GQGISPWSRLCA-STOP
	Intergenic SNP: promoter region *yedY*, promoter region *csrD*	720,258	Chromosome	G	A	*Not applicable*
	yxeP	2,443,607	Chromosome	C	T	A384V
	yxeP	2,443,610	Chromosome	C	G	A385G
	phoQ	3,417,233	Chromosome	G	A	G385S
	afR	4,155,563	Chromosome	G	C	P12R
	lptD	4,659,739	Chromosome	C	G	S646R
KV402	*wcaJ*	865,365	Chromosome	A CAA CAT CTT TAA T	A	222-KIKDV… -> 222-KLDN-STOP
	phoQ	4,702,372	Chromosome	A	T	L210Q
	phoP	4,703,101	Chromosome	C	T	D191N
	Intergenic SNP: promoter region hyp. protein, terminator region *traM*	13,871	Plasmid	A	C	*Not applicable*

[a] The positions that were observed to have mutated through a SNP or indel in the populations cultured in the presence of colistin, compared to the colistin-susceptible strain, are indicated. For each SNP or indel, the strain in which it was found, the associated genes, the location in the specific scaffold of the Illumina/Oxford Nanopore hybrid genome assembly, the reference allele, and the mutant allele are indicated. If the mutation was observed in a coding sequence, the change in the coding sequence of the affected gene is indicated. The residues are numbered according to their position in the reference allele. For mutations not located in a coding sequence, the associated genes are indicated. Hyp. protein; hypothetical protein.

Supplemental Table S3: Overview of location of IS elements.[a]
KP209

IS element	Location		ISMapper result	PCR Results
	Chromosome	Plasmid 1		
IS*1351*	2,273,711 - 2,274,917	None found	Stable	NA
IS*1400*	409,379 - 408,188	None found	Stable	NA
IS*26*	None found	3039 - 3858	Stable	NA
		46,202 - 47,021	Stable	NA
		103,693 - 102,874	Stable	NA
		127,823 - 128,642	Exision	No changes in integration
IS*903B*	None found	124,785 - 125,840	Integration	No changes in integration
IS*Ec52*	None found	90,987 - 92,234	Stable	NA
IS*Kpn1*	533,627 - 535,071	None found	Integration	No changes in integration
	1,298,963 - 1,300,407		Stable	NA
	2,693,035 - 2,691,591		Stable	NA
	3,576,388 - 3,574,944		Stable	NA
	4,229,036 - 4,227,592		Stable	NA
	4,340,825 - 4,339,381		Stable	NA
	4,684,464 - 4,683,020		Exision	No changes in integration
	4,937,819 - 4,936,375		Stable	NA
IS*Kpn26*	None found	29,354 - 28,159	Stable	NA
		51,021 - 49,826	Stable	NA
IS*Sba14*	None found	77,076 - 73,370	Stable	NA

KP040

IS element	Location						ISMapper result	PCR Results
	Chromosome	Plasmid 1	Plasmid 2	Plasmid 3	Plasmid 4	Plasmid 5		
IS*102*	437037						Integration	Novel integration on day 5
		42,230 - 41,183	None found	None found	None found	None found	Stable	NA
		203,355 - 204,410					Stable	NA
		236,546 - 237,601					Stable	NA
IS*186B*	562310 - 563652		None found	None found	None found	None found	Stable	NA
		106,724 - 108,062					Stable	NA
IS*1A*	None found	45,702 - 44,935	None found	None found	None found	None found	Exision	No changes in integration
IS*1B*	None found	147,523 - 148,290	None found	None found	None found	None found	Stable	NA
IS*1F*	None found	185,253 - 186,020	None found	None found	None found	None found	Stable	NA
IS*1R*	3,445,567 - 3,444,800					None found	Stable	NA
	3,444,800 - 3,445,567	None found	None found		None found		Stable	NA
				96,541 - 97,308			Stable	NA
IS*1X3*	None found	249,803 - 249,091	None found	None found			Stable	NA
IS*26*		269,007 - 269,826					Stable	NA
	None found	275,149 - 275,968	None found		None found	None found	Stable	NA
				92,060 - 92,879			Stable	NA
				102,074 - 102,893			Stable	NA
IS*4321R*	None found	214,068 - 215,394	None found	None found	None found	None found	Stable	NA
		236,371 - 235,045					Stable	NA
IS*5*	535,139 - 536,333						Stable	NA
	950,081	None found	None found	None found	None found	None found	Integration	Novel integration on day 1

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IS*903B*	None found	97,940 - 96,884	None found		None found	None found	Stable	NA
		168,847 - 167,793					Stable	NA
		170,660 - 171,716					Stable	NA
				17,990 - 19,038			Stable	NA
IS*Cro1*	None found	294,069 - 296,767	None found	None found	None found	None found	Stable	NA
IS*Ec21*	None found	49,613 - 50,986	None found	None found	None found	None found	Stable	NA
IS*Ehe3*	1,242,490 - 1,243,718	None found	None found	None found	None found	None found	Stable	NA
	1,694,238 - 1,695,466						Stable	NA
	1,753,714 - 1,754,942						Exision	No changes in integration
	3,626,554 - 3,627,782						Stable	NA
IS*Kpn14*	None found	160,642 - 161,409	None found	None found	None found	None found	Stable	NA
IS*Kpn1*	1,860,640 - 1,859,196	None found	None found	None found	None found	None found	Stable	NA
	2,518,008 - 2,519,452						Stable	NA
	2,517,676 - 2,516,232						Stable	NA
	2,577,131 - 2,578,575						Stable	NA
	2,959,264 - 2,960,708						Stable	NA
	3,692,344 - 3,693,788						Stable	NA
	4,311,386 - 4,312,830						Stable	NA
	4,878,025 - 4,876,581						Stable	NA

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IS*Kpn20*	611,247 - 610052						Stable	NA
	706,335 - 705140						Stable	NA
	2,969,913 - 2971108						Stable	NA
	4,319,319 - 4320514		None found		None found	None found	Stable	NA
		282,044 - 283,239					Stable	NA
				7210 - 8405			Stable	NA
				24,339 - 25,534			Stable	NA
IS*Kpn21*	None found	90,829 - 93,081	None found	None found	None found	None found	Stable	NA
		302,918 - 300,666					Stable	NA
IS*Kpn24*	None found	30,990 - 28,537	None found	None found	None found	None found	Stable	NA
IS*Kpn26*	1,611,873 - 1,613,068						Stable	NA
	2,987,253 - 2,988,448						Stable	NA
	5,228,386 - 5,229,581		None found	None found	None found	None found	Stable	NA
		43,574 - 44,769					Stable	NA
		289,171 - 290,366					Stable	NA
IS*Kpn28*	1,686,614 - 1,687,707						Stable	NA
	1,939,240 - 1,938,147						Stable	NA
	2,802,224 - 2,803,317						Stable	NA
	4,289,154 - 4,290,247		None found		None found	None found	Stable	NA
		84,936 - 86,031					Stable	NA
		105,433 - 106,528					Stable	NA
				40,456 - 41,549			Stable	NA

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IS*Kpn38*	1,316,492 - 1,318,089						Stable	NA
		87,674 - 86,077					Stable	NA
		89,232 - 90,829	None found	None found		None found	Stable	NA
		302,919 - 304,516					Integration	No changes in integration
					86,969 - 88,566		Stable	NA
IS*Ppu12*	None found	112,459 - 115,830	None found	None found	None found	None found	Stable	NA
IS*Sen3*	None found	175,514 - 173,559	None found	None found	None found	None found	Stable	NA
IS*Sen4*	None found	280,209 - 278,989	None found	None found	None found	None found	Stable	NA
		292,245 - 293,465					Stable	NA

KP257

	Location					
IS element	**Chromosome**	**Plasmid 1**	**Plasmid 2**	**Plasmid 3**	**ISMapper result**	**PCR Results**
IS*102*	None found	146,179-147,234		None found	Exision	No changes in integration
			28,056 - 29,111		Exision	No changes in integration
IS*1400*	4,888,362 - 4,887,171	None found	None found	None found	Stable	NA
IS*1X3*	None found	133,977 - 134,676	None found	None found	Stable	NA
IS*1X4*	None found	None found	17,567 - 18,334	None found	Stable	NA
IS*903B*	None found	None found	32,740 - 33,789	None found	Stable	NA
			36,985 - 38,034		Exision	No changes in integration
IS*Ec21*	None found	None found	26,032 - 27,405	None found	Stable	NA
IS*Ehe3*	None found	8776 - 7549	None found	None found	Stable	NA
IS*Eic1*	None found	None found	25,486 - 24,728	None found	Stable	NA

Supplemental Table S3 (KP257) continued from page 164

IS*Kpn1*	2,465,446 - 2,464,002	None found	None found	None found	Stable	NA
	4,070,871 - 4,069,427				Stable	NA
	4,120,943 - 4,122,387				Stable	NA
	4,153,758 - 4,152,314				Stable	NA
IS*1S*	None found	None found	50,676 - 51,438	None found	Stable	NA
ISKpn49	None found	15,667 - 18,106	None found	None found	Stable	NA

KV402

	Location				
IS element	**Chromosome**	**Plasmid 1**	**Plasmid 2**	**ISMapper result**	**PCR Results**
IS*1400*	5,029,253 - 5,030,446	None found	None found	Stable	NA

Position of IS elements as determined by ISMapper in the colistin-susceptible and colistin-resistant strains (isolated on the last day of the *in vitro* evolution experiments). The result of ISMapper was classified as excision, novel integration, or stable per IS element. Events were classified compared to the Illumina/Oxford Nanopore hybrid genome assembly of the colistin-susceptible strain. For IS elements with an excision, or novel integration, the changed position was checked through targeted PCRs (Supplemental Figure S1) and Sanger sequencing in all isolates collected during the *in vitro* evolution experiment. Shading indicates that changes were detected through targeted PCR. NA: not applicable.

Supplemental Table S4: Mutations observed in whole genome sequence of axenic colistin-resistant strains.[a]

Strain	Associated feature(s)	Location	Scaffold	Reference	Mutant	Coding change
KP209	*phoQ*	4,336,315	Chromosome	T GGG CGC AGC GTG	T	97-WAQRN -> 97-C
	phoQ	4,336,791	Chromosome	C	A	R256S
KP040	*rho*	3,907,646	Chromosome	C	C GCG ATG TTC TGC	189-QS -> 189-QNIAQS
	wzc	437,037	Chromosome	IS*5* insertion		*Not applicable*
	promoter region of *crrAB* and *crrC*	950,081	Chromosome	IS*102* insertion		*Not applicable*
	Intergenic SNP: promoter region *yedY*, promoter region *csrD*	720,258	Chromosome	G	A	*Not applicable*
	lptD	3,246,640	Chromosome	TAAAA	TAAA	764-ILPYQSSL-STOP -> 764-IYRTRAPCNAAGLQHHPHLRLIEMEKV-STOP
KP257	*phoQ*	3,417,233	Chromosome	G	A	G385S
	lptD	4,659,739	Chromosome	C	G	S646R
KV402	*phoP*	4,703,101	Chromosome	C	T	D191N
	yciM	4,950,035	Chromosome	T	G	V43G
	gcd	409,843	Chromosome	C	A	*Synonymous mutation*

[a] For each strain, the positions that have been mutated between the colistin-susceptible strain, and the colistin-resistant strain picked from the last day of culturing are indicated. For each mutation, the associated feature, the location in the specific scaffold of the Illumina/Oxford Nanopore hybrid genome assembly are indicated, the reference allele and the mutant allele are indicated. If the mutation was observed in a coding sequence, the changes in the coding sequence of the affected gene are indicated. The residues are numbered according to their position in the reference allele. For mutations not located in a coding sequence, the associated coding sequences are indicated.

Supplemental Table S5: Colistin concentrations used during *in vitro* evolution experiments.[a]

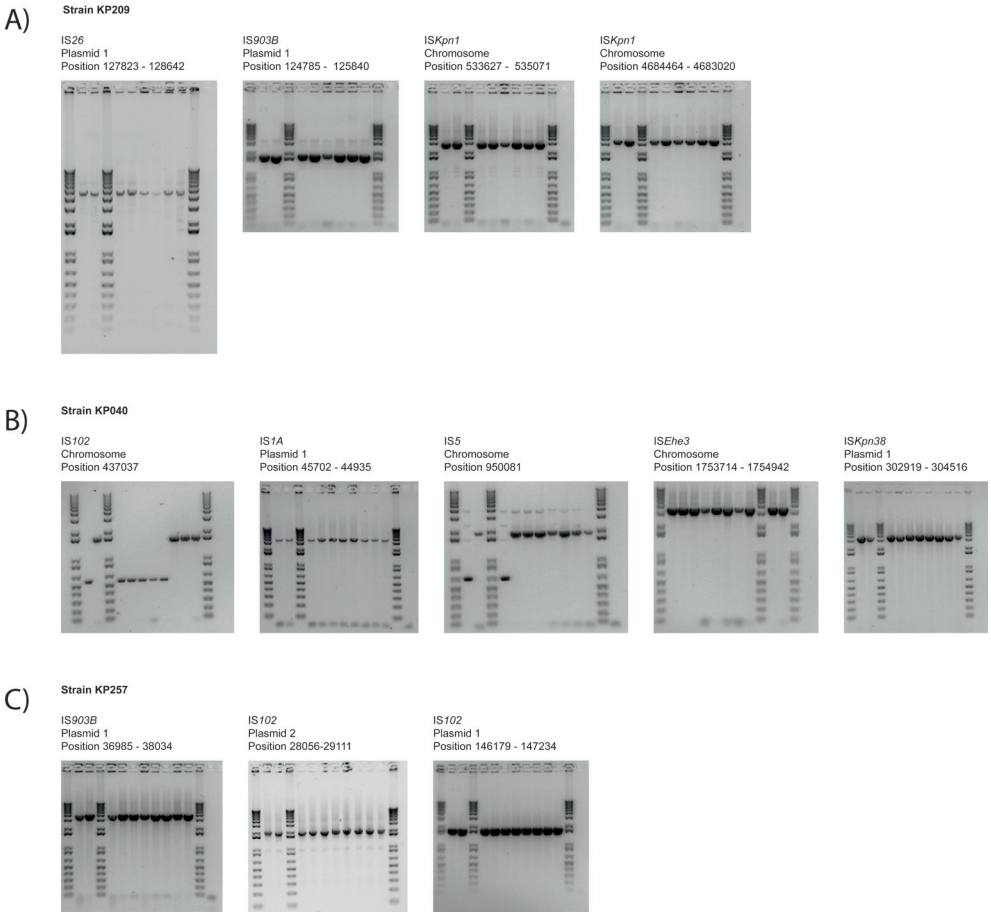
Strain	KP209		KP040		KP257		KV402	
MIC in LB (µg/ml)	4		0.5		2		2	
Day 1	4	8	0.5	1	2	4	2	4
Day 2	4	8	1	2	2	4	2	4
Day 3	8	16	2	4	4	8	4	8
Day 4	16	32	4	8	8	16	8	16
Day 5	32	64	8	16	16	32	16	32
Day 6	32	64	16	32	32	64	32	64
Day 7	64	128	32	64	64	128	64	128

[a] A green background indicates the observation of growth after overnight incubation at 37ºC, red indicates no observable growth. The cultures from which the concentration is underlined, were used to propagate the *in vitro* evolution experiment. If no growth was observed in both cultures, cultures were re inoculated from -80°C stocks of the susceptible strain (only when no growth was observed on day 1), or from cultures stored from the previous day.

Supplemental Table S6: Sequences of oligonucleotide primers.[a]

Name	Sequence (5'3')	Reference
KP209 IS*903B* plasmid 1 124 kbp Fwd	CCT GGA TGA GAG GTA GAT GG	This study
KP209 IS*903B* plasmid 1 124 kbp Rev	GAA GCG TCG TAT CCC ATA AC	
KP209 IS*Kpn1* chromosome 4.6 Mbp Fwd	TGC TGG TGT CGT TTA TCT GC	
KP209 IS*Kpn1* chromosome 4.6 Mbp Rev	ATG GAC AGA TAT CCG CGT TC	
KP209 IS*26* plasmid 1 127 kbp Fwd	GGC AGT GCA AAT CGA AAA AT	
KP209 IS*26* plasmid 1 127 kbp Rev	TCA TTC AGC ACG CAA AAT TC	
KP209 IS*Kpn1* chromosome 533 kbp Fwd	ATG TCG ATC CGA TTC GTC A	
KP209 IS*Kpn1* chromosome 533 kbp Rev	GTT CGT CAG CAC CCA TGT TT	
KP040 IS*Kpn38* plasmid 1 302 kbp Fwd	TCA GGA AAA AGC GAG TCG TT	
KP040 IS*Kpn38* plasmid 1 302 kbp Rev	TTC GTT GAG CGT ATC GAG TG	
KP040 IS*1A* plasmid 1 45 kbp Fwd	CAT GCA GCA TTG GTA CAA CC	
KP040 IS*1A* plasmid 1 45 kbp Rev	ACG TTT TCA ACC CAC AGA GG	
KP040 IS*Ehe3* chromosome 1.7 Mbp Fwd	TGG AGC TGG TAT ACC GGT TC	
KP040 IS*Ehe3* chromosome 1.7 Mbp Rev	CGA CCA GGT GGC AAG TTT AT	
KP040 IS*102* chromosome 437 kbp Fwd	GTC AGT ATT GGA CCA GAT CGT	
KP040 IS*102* chromosome 437 kbp Rev	TGC AAG CAG ACC TTT ATC AAA AT	
KP040 IS*5* chromosome 950 kbp Fwd	GAT TGA TTG CCT GCT CAC CG	
KP040 IS*5* chromosome 950 kbp Rev	ACC AGA CCG AAT GTT ATT GCA	
KP257 IS*903B* plasmid 2 36.9 kbp Fwd	GAA GCC ATG CTG GAT AAG GA	
KP257 IS*903B* plasmid 2 36.9 kbp Rev	GGT CTT AAT GCC CAG CAC AT	
KP257 IS*102* plasmid 1 146 kbp Fwd	ATA TCG CTG AAC CAG TGC TC	
KP257 IS*102* plasmid 1 146 kbp Rev	ACA TCG AGT GGC TTC TGA AA	
KP257 IS*102* plasmid 2 28 kbp Fwd	TGG GTT ACC ACC AAA CGA AT	
KP257 IS*102* plasmid 2 28 kbp Rev	GCT TTC AGA GCC TGG ATG AC	

[a] The name indicates the specific strain and target site of the primer.



Supplemental Figure S7: Agarose gel electrophoresis to determine integration sites of IS elements. PCR reactions were performed to validate the ISMapper results suggesting IS element excision or integration between the colistin-susceptible parental strains and the colistin-resistant strains from the last day of the *in vitro* evolution experiment. PCR reactions were performed using primers spanning the integration site of each IS element (Supplemental Table S2). The 1 kb plus DNA ladder (Thermo Scientific) was used for size comparison. The order of samples in each agarose gel electrophoreses is: 1 kb plus DNA ladder, colistin-susceptible strain, axenic strain of the last day of the *in vitro* evolution experiment, 1 kb plus ladder, colistin-susceptible strain, axenic strain for each day of the *in vitro* evolution experiment, 1 kb plus ladder, negative water control. The order for KP040 IS*Ehe3* was: 1 kb plus marker, colistin-susceptible strain, axenic strain for each day of the *in vitro* evolution experiment, 1 kb plus ladder colistin-susceptible strain, axenic strain for each day of the *in vitro* evolution experiment, 1 kb plus ladder.

Chapter

6 General discussion

Axel B. Janssen

Introduction

Antibiotics are invaluable in modern-day medicine for the treatment and prevention of infections. The indispensability of antibiotics for treatment of infections is emphasized by the increasing rate of antibiotics resistance. The increasing rate of resistance against multiple types of antibiotics in opportunistic Gram-negative bacteria, has led to the revival of colistin as a last-resort drug to treat infections with these multidrug-resistant strains (1–3). The acquisition of colistin resistance in multidrug-resistant Gram-negative bacteria would further hamper the treatment of bacterial infections. Furthermore, colistin resistance could also lower the beneficial effects of the prophylactic selective decontamination of the digestive tract (SDD) treatment, which is used to prevent infections in patients admitted to an intensive care unit (ICU). In addition, studies on the consequences of pan-drug resistance also cite potential difficulties to perform routine medical treatments that require the prophylactic uses of antibiotics like surgeries, caesarean section, and treatment of cancer patients (4). Since diverse species from the Enterobacteriaceae family cause a significant portion of clinical infections, development of colistin resistance in multidrug-resistant Enterobacteriaceae would be of particular concern (5, 6).

Since the revival of colistin as a drug of last-resort, there has been a marked interest in the mechanisms of colistin resistance. As colistin had previously fallen into disuse due to its toxic side-effects, there is a gap in the knowledge on the mechanisms of resistance in Enterobacteriaceae, and the evolutionary trajectories through which these may occur. In this thesis, we have addressed this gap by investigating the mechanisms and evolution of colistin resistance in Enterobacteriaceae isolated from diverse clinical settings.

Mechanisms of colistin resistance in clinical Enterobacteriaceae isolates

Enterobacteriaceae are well-known opportunistic pathogens that can cause infections in both immunocompromised patients and in the healthy population. *Escherichia coli* is specifically known to cause a significant portion of the bloodstream infections observed worldwide (5). As the prevalence of multidrug resistance in *E. coli* isolates is increasing (5, 7), these infections are increasingly harder to treat. The acquisition of colistin resistance in *E. coli* bloodstream isolates would further threaten the treatment of these infections. In **Chapter 2**, we aimed to gained insight into the prevalence, and the mechanisms of colistin resistance in bloodstream *E. coli* isolates. By retrospective screening for colistin resistance in all bloodstream *E. coli* strains isolated during a ten-year period (1140 in total) in

the University Medical Center Utrecht (UMC Utrecht) in Utrecht, the Netherlands, we found ten colistin-resistant *Escherichia* isolates. Three of the ten patients which these ten strains were obtained, were treated with colistin in the three months before isolation of the isolate. The prevalence of colistin resistance (0.88%) that we observed in bloodstream isolates, was similar to previous reports (8-13). The limited prevalence we have observed, might stem from the rare use of colistin and consequently the low exposure of *E. coli* to this antibiotic in Dutch hospitals. Colistin use is low due to the low prevalence of carbapenem resistance (2).

We observed that the colistin-resistant bloodstream *Escherichia* isolates were genomically not closely related. This signified the independent *de novo* acquisition of colistin resistance in these strains. Three strains belonged to the nosocomial successful multidrug-resistant C clade of the ST131 lineage of *E. coli* (14). In one strain colistin resistance was mediated by the acquisition of a mobile colistin resistance (*mcr*)-*1.1*-gene encoding IncX4-type plasmid. The global dissemination of this plasmid-type, and this exact plasmid in particular, which has been found in *K. pneumoniae* (15), *E. coli* (16), and *Salmonella enterica* (17, 18) strains, illustrates the ability of plasmids harbouring *mcr-1* to efficiently spread amongst Enterobacteriaceae.

The discovery of the first of the *mcr* genes in 2015 has sparked a flurry of reports on the mechanism, occurrence, and spread of this type of colistin resistance. Although the initial mobilization event of *mcr*-1 by IS*Apl1* elements has been relatively recent (19), plasmids encoding *mcr*-1 and other *mcr* genes have disseminated globally, indicating the success of these plasmids in spreading between strains. Since we find a low prevalence of *mcr*-positive strains in our studies, the occurrence and dissemination of this mechanism of colistin resistance in the Netherlands does not seem of concern. Whilst *mcr* genes seem an important mechanism of colistin resistance in *E. coli* (20), we observed nine *mcr*-negative colistin-resistant *E. coli* isolates. We unravelled the mechanisms of colistin resistance in these strains through a combination of comparative genomics and construction of chromosomal transgene insertion mutants. In absence of colistin-susceptible isogenic strains, we were able to experimentally determine that variants of BasR (G53S), and BasS (L10R, A159P, and duplication of the HAMP-domain) could contribute to colistin resistance in four strains. Even though we found that multiple mechanisms led to colistin resistance in five of the ten obtained isolates, we observed that all ten colistin-resistant strains produced lipid A modified with phosphoethanolamine (PEtN). The observation of PEtN modification of lipid A in all strains suggests that increased activity of a lipid A PEtN transferase is responsible for colistin resistance in these strains.

The development of colistin resistance in infectious Gram-negative bacteria could

hamper the treatment of infections. However, colistin use is not limited to curative treatments. SDD treatment includes colistin and other types of antibiotics, to reduce 28-day mortality and morbidity in ICU patients through the selective eradication of a part of the gut microbiota, including Enterobacteriaceae. The use of antibiotics in SDD has been observed to not affect the overall rate of antibiotic resistance (21). However, SDD has become increasingly controversial for its use of last-resort antibiotics as prophylactic agents (22, 23). Not only are the beneficial effects of SDD only observed in countries with a relatively low rate of antibiotic resistance (e.g. the Netherlands, Germany, and France) (24), the concurrent use of colistin, tobramycin, and a third-generation cephalosporin can result in the selection and spread of multidrug-resistant Gram-negative bacteria (25–28). Since the success of SDD relies on the ability to selectively eradicate opportunistic pathogens from the gut microbiota, resistance would threaten the effectiveness of SDD in countries with a low rate of antibiotic resistance. Therefore, the clinical guidelines of SDD in the Netherlands dictate that surveillance of resistance against the antibiotics included in SDD through rectal swabs of patients receiving this treatment, is necessary (29). In **Chapter 3** and **Chapter 4,** we used the strains collected by this routine surveillance (27, 30) to investigate the spread, mechanisms, and evolution of colistin resistance in Enterobacteriaceae in ICU-settings.

In **Chapter 3**, we examined the colistin-resistant strains isolated from five ICU-admitted patients receiving SDD in the UMC Utrecht (30). In one patient, we observed persistent gut colonisation by a colistin-resistant *E. coli* strain harbouring aminoglycoside and third-generation cephalosporin resistance genes. This persistent colonisation defies the purpose of SDD, and reflects the incidental reports on the effects of SDD on selection for multidrug-resistant strains (25, 27). The persistently colonising *E. coli* strain developed resistance through two separate evolutionary pathways by developing a G53A substitution in BasR, or an 18 bp deletion in *basS*. An additional I424S substitution in YrfF in three of the four isolates harbouring the 18 bp deletion in *basS* probably led to a mucoid phenotype. Since capsule polysaccharides are known to play a role in evasion of the immune system (31), the effects of this phenotype on *in vivo* fitness should be investigated. Both the 18 bp deletion in *basS* and the substitution in YrfF, led to a lower maximum specific growth rate *in vitro*. The *in vivo* evolution of colistin resistance in an ESBL-producing, aminoglycoside-resistant *E. coli* strain may serve as a cautionary tale that SDD can select for multidrug resistance even in countries with low levels of antibiotic resistance, like the Netherlands.

In four other ICU-admitted patients, we observed transient gut colonisation by colistin-resistant *Klebsiella aerogenes*, *Enterobacter asburiae*, and *E. coli* strains. In

one *E. coli* strain, we observed the presence of *mcr-1.1*. In another *E. coli* strain, we observed a G19E substitution in BasR contributing to colistin resistance. The *E. coli* strains isolated from different patients were genomically not closed related. We observed *in vivo* evolution of colistin resistance in *K. aerogenes* through a G53S PmrA substitution by comparing a colistin-susceptible, and the colistin-resistant strains isolated from the one patient. Furthermore, we identified an *E. asburiae* strain with high-level colistin resistance (minimal inhibitory concentration (MIC) of 8192 µg/ml) which warrants further work, as resistance to such high colistin concentrations has not been reported often in clinical isolates (32), and may yield surprising new insights into mechanisms of colistin resistance.

In line with the successful gut colonisation by a ESBL-producing, aminoglycoside-resistant *E. coli* strain observed in **Chapter 3**, we also described how a multidrug-resistant *K. pneumoniae* strain developed colistin resistance through multiple evolutionary pathways, during outbreak in an ICU where patients receive SDD, in **Chapter 4**. Here, we analysed strains isolated during an outbreak caused by an ESBL-producing, aminoglycoside-resistant *K. pneumoniae* strain. Rather than to curb this strain (27), the introduction of SDD in this particular ICU led to the independent acquisition of colistin resistance in multiple isolates of the outbreak strain. Thus, the use of colistin in SDD led to the selection of the extensively drug resistant strains. In these colistin-resistant strains, we observed that multiple unique mutations in *yciM*, *phoQ* and *lpxM*, and either disruption by insertion sequence (IS) elements, or complete loss, of *mgrB* contributed to colistin resistance.

In vitro evolution of *K. pneumoniae* complex strains towards colistin resistance

In **Chapters 2**, **3**, and **4**, we investigated the mechanisms and evolution of colistin resistance by collecting colistin-resistant Enterobacteriaceae from clinical settings. Collection of colistin-resistant strains limited the ability to investigate the evolutionary pathways that had led to colistin resistance. To investigate the evolutionary pathways and dynamics within a population during development of colistin resistance, we performed an *in vitro* evolution experiment using four clinical, colistin-susceptible, *K. pneumoniae* complex strains in **Chapter 5**. Through population sequencing of cultures grown under the selective pressure of increasing colistin concentrations during an *in vitro* evolution experiment, we found that the mutations leading to colistin resistance were selectively and swiftly fixed within the populations. We observed a combination of single nucleotide polymorphisms (SNPs), insertions and deletions (indels), and novel integration of IS elements in

genes associated with LPS biosynthesis and modification, and capsule structures. The lipid A moieties of the colistin-resistant *K. pneumoniae* complex strains were modified through a combination of hydroxylation, palmitoylation, and the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N). The development of colistin resistance, had a variable effect on virulence characteristics, as we observed a decrease in susceptibility to the human cathelicidin LL-37 in three colistin-resistant strains, but increased susceptibility to serum in two. In a *C. elegans* virulence model we observed an increased virulence of two of the colistin-resistant *K. pneumoniae* complex strains, compared to their colistin-susceptible parental strain. These results reflect the evolution of colistin resistance through multiple evolutionary pathways we have observed in the colistin-resistant strains isolated from clinical settings, and imply the swift development and fixation of beneficial mutations under selective pressure of colistin.

Multiple evolutionary pathways towards colistin resistance.

The *de novo* occurrence of mutations in genes encoding LPS modification and biosynthesis, and capsule synthesis systems, appear to be an important mechanism through which colistin resistance can develop in Enterobactericeae. Throughout this thesis, we have observed that colistin resistance in genetically diverse Enterobacteriaceae can develop through a range of mutations in a number of the genes related to these systems. Even in clonally related isolates from an outbreak of an ESBL-producing, aminoglycoside-resistant *K. pneumoniae* strain in an ICU, we observed that *de novo* development of colistin resistance in each isolate had occurred through unique mutations.

In the isolates we investigated, we observed that most mutations that contributed to colistin resistance were associated with the PhoPQ, PmrAB (BasRS in *E. coli*), or CrrAB two-component regulatory systems, which all play a role in regulating expression of genes involved in LPS modification. Amongst these two-component regulatory systems, PmrAB seems to fulfil the central role, because of its control over the transcriptional activity of the genes responsible for the modification of lipid A with cationic groups (L-Ara4N and PEtN) that we observed in our studies. The ability of PhoPQ and CrrAB to activate PmrAB through PmrD and CrrC respectively, emphasizes this critical role of PmrAB. The central role of PmrAB suggests that this two-component regulatory system might be an interesting target for the development of an inhibitor, to reduce the expression of the genes responsible for the modification of lipid A with cationic groups.

Within the PmrAB two-component regulatory system, the substitution of specific

residues seems sufficient to cause colistin resistance. Within the response regulator PmrA, particularly frequent observed substitutions are the G53 and R81 residues that are located in the receiver domain of this transcriptional regulator. Structural studies have shown that G53 is located in the active site of PmrA near the phosphorylation site, and plays a role in binding of PmrD. The R81 residue is located at the interaction site between PmrA with PmrD. Substitution of G53 might lead to steric hindrance of the phosphatase activity of PmrA. Both substitution of G53 and R81 might lead to an increased binding of PmrD, to keep if from dephosphorylating (33). However, not all the individual components of the PhoPQ, PmrAB, and CrrAB two-component regulatory systems have been investigated through structural studies. Such studies might lead to interesting new insights into the exact mechanisms behind the ability of some of specific substitutions frequently observed to cause colistin resistance.

In addition to investigating the role of non-synonymous mutations in development of colistin resistance, we hypothesized in this thesis, that the novel integration of IS elements can lead to colistin resistance as well. In the *in vitro* evolution experiment, we observed that the integration of an IS*5* element in the promoter region of *crrAB* and *crrC* coincides with the increase in MIC of colistin. In addition, we observed that inactivation of *wzc* by an IS*102* insertion occurred at the same time as an increase in MIC of colistin. Although the role of IS elements in colistin resistance is well-described when it comes to inactivation of *mgrB* (34–36), the role of IS elements outside *mgrB* inactivation are poorly understood. IS elements can act on gene expression through the inactivation of genes by integrating into coding sequences, but also through integration of IS elements in the promoter region of genes thereby changing the expression levels of the genes associated that promoter region (37, 38). Additional experimentation through functional genomics, including transposon sequencing and sequencing of the transcriptome, might give more insight in these mechanisms. When a novel excision or integration of an IS element is hypothesized to cause colistin resistance in a wildtype strain, verification of the ability to cause resistance might be difficult. The construction of a targeted mutant for the observed IS element integration or excision is presumably more difficult than for SNPs and indels, because of the large size of IS elements, and their ability to transpose independently.

Besides the role of IS elements, and the ability of non-synonymous mutations to cause colistin resistance, the role of synonymous mutations in the development of colistin resistance can be an interesting future research topic. Synonymous mutations may affect susceptibility to antibiotics by affecting gene expression through differences in mRNA stability and translation rate, which may lead to colistin resistance (39).

In addition to mutations in two-component regulatory systems, other novel mechanisms of colistin resistance should be investigated further as well. The role of capsular polysaccharides in the development of colistin resistance has been reported (40, 41), but remains poorly studied otherwise. We observed mutations in genes associated with capsular polysaccharides in a number of strains, through the inactivation of *wzc* by the insertion of IS*102*, but also non-synonymous mutations in *wcaJ*, and *yrfF*. Both *wcz* and *wcaJ* play a direct role in the synthesis of capsular polysaccharides, whilst *yrfF* is involved in the regulation of the capsular polysaccharide pathway. It is important to note that if mutations leading to changes to the expression of capsular polysaccharides contribute to colistin resistance, these mutations can also have an important impact on virulence characteristics, as capsule polysaccharides are known to play a role in evasion of the immune system (31).

While we found that multiple mutations may contribute to colistin resistance, we also observed that mutations that lead to colistin can have variable collateral effects. For example, in our *in vitro* evolution experiment, we observed mutations in *phoPQ* in three of the four *K. pneumoniae* complex strains we evolved towards colistin resistance. In these three strains however, we observe variable effects on the virulence characteristics. Thus, the genetic background of a particular strain that develops colistin resistance through *de novo* mutations, seems to play an important role in the exact effects this mutation will have. Supporting this are the observations in our *E. coli* experiments, in which we do not observe the same levels of colistin resistance in our BW27848 transgene insertion mutants, as in the colistin-resistant nosocomial strains. Thus, predicting whether an observed variation can lead to colistin resistance, and to what exact MIC, seems difficult based on genome sequence analysis alone.

To understand the effects a mutation may have on a bacterium, construction of mutant strains is crucial. Yet, construction of mutations in the clinical isolates where a mutation might be found, is often difficult. Nosocomial strains often harbour a collection of plasmids encoding for acquired antibiotic resistance genes, making them multidrug-resistant. In addition, if a plasmid-based system is used for constructions of mutants, like the pGRG36-based Tn*7* transposon system (42) we used to construct the transgene insertion mutants, plasmid incompatibility may also affect the construction of mutants. Clearing plasmids from these nosocomial multidrug-resistant strains might offer a solution to this problem, by repeated growth without in absence of a selective pressure for these plasmid. Other methods to target the plasmids, including CRISPR-Cas9 may solve these problems (43). Other systems to make targeted mutations in these multidrug-resistant clinical strains can include P1 phage transduction (44), and

systems relying on recombination (45). Besides plasmids, phenotypic variations in clinical strains (e.g. through expression of capsular polysaccharides), can also make the construction of mutations more difficult, by inhibiting the uptake of mobile genetic elements. These combined difficulties can result in the resorting to lab-adapted strains for the functional analysis of mutations.

Phenotypic testing for colistin resistance is challenging. Automated susceptibility testing systems (e.g. Vitek (bioMérieux), or Phoenix (BD)) have been found to be imperfect methods to determine colistin susceptibility (46–49). These oft used automated systems may lead to both false-susceptible, but also false-resistant, determinations of colistin resistance. Other phenotypic tests that are frequently used, like Etests or disc diffusion assays, cannot be performed for colistin, since the cationic colistin has difficulties diffusing through agar. Because colistin has regained its clinical significance, a joint EUCAST and CLSI committee has specified that only broth microdilution methods should be performed for colistin susceptibility testing. However, broth microdilution methods for colistin susceptibility testing are traditionally plagued by difficulties (50, 51). Therefore, the EUCAST and CLSI have specified a detailed method that should be used, making this method complex and time-consuming to perform. Especially in settings with a high prevalence of colistin resistance, the difficulties in colistin susceptibility testing may have a significant impact on clinical decision making. In addition, we observed that there are difficulties in distinguishing closely related species from *Escherichia* and *Klebsiella*, in routine diagnostic matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) species identification (52–54). The difficulties in species identification adds to the complexity of predicting whether a mutation might yield colistin resistance, as different species might have different regulatory circuits leading to colistin resistance.

The impact of colistin resistance in clinical settings

Through the collection of the colistin-resistant Enterobacteriaceae from bloodstream infections, and from ICU-admitted patients who received SDD treatment, we observed a low prevalence of colistin resistance in Enterobacteriaceae in clinical isolates from the UMC Utrecht. Thus, the curative and prophylactic use of colistin, does not seem to be directly hampered by this low prevalence of colistin resistance. However, we have observed that when a strain is resistant to the third-generation cephalosporin and tobramycin used in SDD, colistin alone is not fully successful in eradicating this strain. In contrast, it can lead to the successful and prolonged gut colonization with a colistin- and aminoglycoside-resistant, ESBL-producing strain. In addition, it can lead to

an already successful multidrug-resistant outbreak strain, to develop colistin resistance, further hampering treatment. This emphasizes the need for thorough surveillance during SDD, to detect and thwart any possible outbreaks with multidrug-resistant strains.

In contrast to the Netherlands, a considerably higher prevalence of colistin resistance amongst clinical Enterobacteriaceae isolates has been reported in other countries, including Greece (55), Italy (56, 57), and Brazil (58). This higher prevalence will significantly impact the use of colistin as a last-resort antibiotic. In the case of a high background rate of antibiotic resistance, specifically to third-generation cephalosporins and aminoglycosides, the use of SDD can contribute to the further development of colistin resistance in already multidrug-resistant pathogens. The observation of independent *de novo* acquisition of resistance in some of the non-clonally related isolates we have analysed, through multiple unique mutations, without evidence for exposure to colistin, does not bode well for the treatment of infections caused by multidrug-resistant Enterobacteriaceae

When the use of colistin for the treatment of infections with multidrug-resistant Gram-negative bacteria increases, the morbidity due to the toxic side-effects will potentially also increase. However, with the revival of colistin, efforts to reduce the toxic side-effects during use of colistin in patients are being made. Since the toxic side-effects are being observed in a dose-, and duration-dependent manner (59), optimisations to the dosing of colistin treatment may reduce toxic side-effects. To this aim, the use of loading-dose, to shorten the time to achieve an effective concentration in patients, has been explored (60). In addition, the effects of improved hydration, alkalinisation of the urine (61), and administration of inhibitors for nephron reabsorption (62) have been investigated. With these developments, the use of colistin should be safer in clinical settings.

However, even with innovations to colistin treatment, the emergence of colistin resistance will make infections with multidrug-resistant Gram-negative bacteria increasingly difficult to treat. Since the development of novel antibiotics is currently short of compounds with a novel mechanism of action (63, 64), alternative measures than antibiotic therapy to eradicate infecting bacteria are actively being sought. These alternatives include: the use of (products of) bacteriophages (65, 66), antimicrobial peptides (67), antibody-mediated therapy (68), molecules that activate the humoral complement system (68), and novel compounds that block resistance mechanisms against antibiotics (69–71). Most of these treatments, however, are still experimental, and may run into problems upon *in vivo* use, including through stability, efficacy, and specificity issues. In addition, these novel treatments also still need the same extensive, and expensive, clinical trials which are one of the reasons that the antibiotic development pipeline has been

depleted. Without new antibiotics, stopping the spread of multidrug-resistant Gram-negative bacteria may be an efficient method to reduce the incidence of infections caused by these organisms. Most of these measures will come through hospital hygienic measures, including hand hygiene, contact precautions, and increased screening efforts, which can result in a lower overall rate of resistance in an hospital (72).

Since antibiotic therapy is crucially important for medicine, the threat of non-treatable infections has prompted governmental organisations to find new ways to stimulate innovation. In addition to the creation and funding of public-private partnerships, new ways to stimulate pharmaceutical companies to develop novel antibiotics are being explored. Although financial compensation for the development costs, or alternative reimbursement models for novel antibiotics could lower the burden for the pharmaceutical companies developing the drug (73), this might not solve the inherent difficulties of finding a new antibiotic. Other regulatory measures to safeguard antibiotics include the increased attention for the appropriate use of antibiotics in both human and veterinary medicine, stimulating the development of infection prevention and control programs, and strengthening of antibiotic resistance surveillance efforts (74–76). These measures should contribute to the appropriate use of antibiotics in the near future.

For colistin specifically, the discovery of the first of the *mcr* genes in 2015 has led to a major shift in the regulations on the use of colistin. As colistin was previously widely used in veterinary medicine, while becoming increasingly vital as a last-resort drug in human medicine, its use in veterinary use has now been severely restricted. These restrictions include the banning of colistin as growth promoter, and its use to treat enteric infections (77, 78). These restrictions are all designed to safeguard colistin as a drug of last-resort for human medicine.

Conclusion

The surging rate of antibiotic resistance has compelled the international healthcare community to reconsider its antibiotic policies. As a result, antibiotics are used more conservative and the lack in development of novel antibacterial agents is slowly being addressed. However, the proper use of antibiotics in human and veterinary medicine should remain a top priority in the foreseeable future nonetheless, as the threat of extensively drug-resistant pathogens still remains. The urge for additional research into the basic mechanisms and spread of antibiotic resistance amongst nosocomial relevant pathogens should thus not diminish. Additional research may lead to the discovery of presently unknown

mechanisms of colistin resistance, and the identification of novel targets for the development of compounds that may block colistin resistance from developing.

References

1. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, Goossens H, Laxminarayan R. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci 115:E3463–E3470.
2. European Centre for Disease Prevention and Control. 2019. Surveillance of antimicrobial resistance in Europe 2018Surveillance of antimicrobial resistance in Europe.
3. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP. 2013. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infect Dis 13:785–796.
4. Smith R, Coast J. 2013. The true cost of antimicrobial resistance. Br Med J 346:f1493.
5. Diekema DJ, Hsueh P-R, Mendes RE, Pfaller MA, Rolston K V., Sader HS, Jones RN. 2019. The microbiology of bloodstream infection: 20-year trends from the SENTRY antimicrobial surveillance program. Antimicrob Agents Chemother 63:e00355-19.
6. de Kraker MEA, Jarlier V, Monen JCM, Heuer OE, van de Sande N, Grundmann H. 2013. The changing epidemiology of bacteraemias in Europe: trends from the European Antimicrobial Resistance Surveillance System. Clin Microbiol Infect 19:860–868.
7. World Health Organization. 2014. Antimicrobial resistance. Global report on surveillance.
8. Kim YA, Yong D, Jeong SH, Lee K. 2017. Colistin resistance in *Escherichia coli* isolates from patients with bloodstream infection in Korea. Ann Lab Med 37:172–173.
9. Quan J, Li X, Chen Y, Jiang Y, Zhou Z, Zhang H, Sun L, Ruan Z, Feng Y, Akova M, Yu Y. 2017. Prevalence of *mcr-1* in *Escherichia coli* and *Klebsiella pneumoniae* recovered from bloodstream infections in China: a multicentre longitudinal study. Lancet Infect Dis 17:400–410.
10. Wang X, Liu Y, Qi X, Wang R, Jin L, Zhao M, Zhang Y, Wang Q, Chen H, Wang H. 2017. Molecular epidemiology of colistin-resistant Enterobacteriaceae in inpatient and avian isolates from China: high prevalence of *mcr*-negative *Klebsiella pneumoniae*. Int J Antimicrob Agents 50:536–541.
11. Corbella M, Mariani B, Ferrari C, Comandatore F, Scaltriti E, Marone P, Cambieri P. 2017. Three cases of *mcr-1*-positive colistin-resistant *Escherichia coli* bloodstream infections in Italy, August 2016 to January 2017. Eurosurveillance 22:pii=30517.
12. Principe L, Piazza A, Mauri C, Anesi A, Bracco S, Brigante G, Casari E, Agrappi C, Caltagirone M, Novazzi F, Migliavacca R, Pagani L, Luzzaro F. 2018. Multicenter prospective study on the prevalence of colistin resistance in *Escherichia coli*: relevance of *mcr-1*-positive clinical isolates in Lombardy, northern Italy. Infect Drug Resist 11:377–385.
13. Zhong YM, Liu WE, Zheng ZF. 2019. Epidemiology and molecular characterization of *mcr-1* in *Escherichia coli* recovered from patients with bloodstream infections in Changsha, central China. Infect Drug Resist 12:2069–2076.
14. McNally A, Kallonen T, Connor C, Abudahab K, Aanensen DM, Horner C, Peacock SJ, Parkhill J, Croucher NJ, Corander J. 2019. Diversification of colonization factors in a multidrug-resistant *Escherichia coli* lineage evolving under negative frequency-dependent selection. mBio 10:e00644-19.
15. Di Pilato V, Arena F, Tascini C, Cannatelli A, Henrici De Angelis L, Fortunato S, Giani T, Menichetti F, Rossolini GM. 2016. *mcr-1.2*, a new *mcr* variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing *Klebsiella pneumoniae* strain of sequence type 512. Antimicrob Agents Chemother 60:5612–5615.
16. Ageevets V, Lazareva I, Mrugova T, Gostev V, Lobzin Y, Sidorenko S. 2019. IncX4 plasmids harbouring *mcr-1* genes: further dissemination. J Glob Antimicrob Resist 18:166–167.
17. Lu X, Zeng M, Xu J, Zhou H, Gu B, Li Z, Jin H, Wang X, Zhang W, Hu Y, Xiao W, Zhu B, Xu X, Kan B. 2019. Epidemiologic and genomic insights on *mcr-1*-harbouring *Salmonella* from diarrhoeal outpatients in Shanghai, China, 2006–2016. EBioMedicine 42:133–144.
18. Carroll LM, Zurfluh K, Jang H, Gopinath G, Nüesch-Inderbinen M, Poirel L, Nordmann P, Stephan R, Guldimann C. 2018. First report of an *mcr-1*-harboring *Salmonella enterica* subsp. *enterica* serotype 4,5,12:i:- strain isolated from blood of a patient in Switzerland. Int J Antimicrob Agents 52:740–741.
19. Wang R, van Dorp L, Shaw LP, Bradley P, Wang Q, Wang X, Jin L, Zhang Q, Liu Y, Rieux A, Dorai-Schneiders T, Weinert LA, Iqbal Z, Didelot X, Wang H, Balloux F. 2018. The global distribution and spread of the mobilized colistin resistance gene *mcr-1*. Nat Commun 9:1179.
20. Elbediwi M, Li Y, Paudyal N, Pan H, Li X, Xie S, Rajkovic A, Feng Y, Fang W, Ranking SC, Yue M. 2019. Global

burden of colistin-resistant bacteria: mobilized colistin resistance genes study. Microorganisms 7:461.
21. Wittekamp BHJ, Oostdijk EAN, Cuthbertson BH, Brun-Buisson C, Bonten MJM. 2019. Selective decontamination of the digestive tract (SDD) in critically ill patients: a narrative review. Intensive Care Med.
22. Timsit JF, Bassetti M. 2018. Antipathy against SDD is justified: Yes. Intensive Care Med 44:1165–1168.
23. Bastin AJ, Ryanna KB. 2009. Use of selective decontamination of the digestive tract in United Kingdom intensive care units. Anaesthesia 64:46–49.
24. Wittekamp BH, Plantinga NL, Cooper BS, Lopez-Contreras J, Coll P, Mancebo J, Wise MP, Morgan MPG, Depuydt P, Boelens J, Dugernier T, Verbelen V, Jorens PG, Verbrugghe W, Malhotra-Kumar S, Damas P, Meex C, Leleu K, Van Den Abeele AM, Gomes Pimenta De Matos AF, Fernández Méndez S, Vergara Gomez A, Tomic V, Sifrer F, Villarreal Tello E, Ruiz Ramos J, Aragao I, Santos C, Sperning RHM, Coppadoro P, Nardi G, Brun-Buisson C, Bonten MJM. 2018. Decontamination strategies and bloodstream infections with antibiotic-resistant microorganisms in ventilated patients: a randomized clinical trial. J Am Med Assoc 320:2087–2098.
25. Lübbert C, Faucheux S, Becker-Rux D, Laudi S, Dürrbeck A, Busch T, Gastmeier P, Eckmanns T, Rodloff AC, Kaisers UX. 2013. Rapid emergence of secondary resistance to gentamicin and colistin following selective digestive decontamination in patients with KPC-2-producing *Klebsiella pneumoniae*: a single-centre experience. Int J Antimicrob Agents 42:565–570.
26. Tascini C, Sbrana F, Flammini S, Tagliaferri E, Arena F, Leonildi A, Ciullo I, Amadori F, Di Paolo A, Ripoli A, Lewis R, Rossolini GM, Menichetti F, Lambelet P, Forfori F, Viaggi B, Papini F, Urbani L, Malacarne P. 2014. Oral gentamicin gut decontamination for prevention of KPC-producing *Klebsiella pneumoniae* infections: relevance of concomitant systemic antibiotic therapy. Antimicrob Agents Chemother 58:1972–1976.
27. Halaby T, Al Naiemi N, Kluytmans J, van der Palen J, Vandenbroucke-Grauls CMJE. 2013. Emergence of colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. Antimicrob Agents Chemother 57:3224–3229.
28. Buelow E, Gonzalez TB, Versluis D, Oostdijk EAN, Ogilvie LA, van Mourik MSM, Oosterink E, van Passel MWJ, Smidt H, D'Andrea MM, de Been M, Jones B V., Willems RJL, Bonten MJM, van Schaik W. 2014. Effects of selective digestive decontamination (SDD) on the gut resistome. J Antimicrob Chemother 69:2215–2223.
29. Oostdijk EAN. 2019. Selective decontamination: SWAB guideline revised. Netherlands J Crit Care 27:26–27.
30. van Hout D, Janssen AB, Rentenaar RJ, Vlooswijk JPM, Boel CHE, Bonten MJM. 2019. The added value of the selective SuperPolymyxin™ medium in detecting rectal carriage of Gram-negative bacteria with acquired colistin resistance in intensive care unit patients receiving selective digestive decontamination. Eur J Clin Microbiol Infect Dis.
31. Llobet E, Tomás JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154:3877–3886.
32. Doi Y, Husain S, Potoski BA, McCurry KR, Paterson DL. 2009. Extensively drug-resistant *Acinetobacter baumannii*. Emerg Infect Dis 15:980–982.
33. Luo SC, Lou YC, Rajasekaran M, Chang YW, Hsiao CD, Chen C. 2013. Structural basis of a physical blockage mechanism for the interaction of response regulator PmrA with connector protein PmrD from *Klebsiella pneumoniae*. J Biol Chem 288:25551–25561.
34. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Türkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. J Antimicrob Chemother 70:75–80.
35. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, the COLGRIT Study Group, Vatopoulos A, Rossolini GM. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob Agents Chemother 58:5696–5703.
36. Halaby T, Kucukkose E, Janssen AB, Rogers MRC, Doorduijn DJ, van der Zanden AGM, al Naiemi N, Vandenbroucke-Grauls CMJE, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. Antimicrob Agents Chemother 60:6837–6843.
37. Vandecraen J, Chandler M, Aertsen A, Van Houdt R. 2017. The impact of insertion sequences on bacterial genome plasticity and adaptability. Crit Rev Microbiol 43:709–730.
38. Schnetz K, Rak B. 1992. IS*5*: a mobile enhancer of transcription in *Escherichia coli*. Proc Natl Acad Sci

89:1244–1248.
39. Agashe D, Sane M, Phalnikar K, Diwan GD, Habibullah A, Martinez-Gomez NC, Sahasrabuddhe V, Polachek W, Wang J, Chubiz LM, Marx CJ. 2016. Large-effect beneficial synonymous mutations mediate rapid and parallel adaptation in a bacterium. Mol Biol Evol 33:1542–1553.
40. Campos MA, Vargas MA, Regueiro V, Llompart CM, Albertí S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun 72:7107–7114.
41. Pal S, Verma J, Mallick S, Rastogi SK, Kumar A, Ghosh AS. 2019. Absence of the glycosyltransferase WcaJ in *Klebsiella pneumoniae* ATCC13883 affects biofilm formation, increases polymyxin resistance and reduces murine macrophage activation. Microbiology 165:891–904.
42. McKenzie GJ, Craig NL. 2006. Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn*7* without need for selection of the insertion event. BMC Microbiol 6:39.
43. Buckner MMC, Ciusa ML, Piddock LJV. 2018. Strategies to combat antimicrobial resistance: anti-plasmid and plasmid curing. FEMS Microbiol Rev fuy031:781–804.
44. Thomason LC, Costantino N, Court DL. 2007. *E. coli* genome manipulation by P1 transduction. Curr Protoc Mol Biol Unit 1.17.
45. Heermann R, Zeppenfeld T, Jung K. 2008. Simple generation of site-directed point mutations in the *Escherichia coli* chromosome using Red®/ET® Recombination. Microb Cell Fact 7.
46. Dafopoulou K, Zarkotou O, Dimitroulia E, Hadjichristodoulou C, Gennimata V, Pournaras S, Tsakris A. 2015. Comparative evaluation of colistin susceptibility testing methods among carbapenem-nonsusceptible *Klebsiella pneumoniae* and *Acinetobacter baumannii* clinical isolates. Antimicrob Agents Chemother 59:4625–4630.
47. Jayol A, Nordmann P, Lehours P, Poirel L, Dubois V. 2018. Comparison of methods for detection of plasmid-mediated and chromosomally encoded colistin resistance in Enterobacteriaceae. Clin Microbiol Infect 24:171–174.
48. Matuschek E, Åhman J, Webster C, Kahlmeter G. 2018. Antimicrobial susceptibility testing of colistin - evaluation of seven commercial MIC products against standard broth microdilution for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. Clin Microbiol Infect 24:865–870.
49. Pfennigwerth N, Kaminski A, Korte-Berwanger M, Pfeifer Y, Simon M, Werner G, Jantsch J, Marlinghaus L, Gatermann SG. 2019. Evaluation of six commercial products for colistin susceptibility testing in Enterobacterales. Clin Microbiol Infect 25:1385–1389.
50. Satlin MJ, Weinstein MP, Patel J, Romney M, Kahlmeter G, Giske CG, Turnidge J. 2020. Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) position statements on polymyxin B and colistin clinical breakpoints. Clin Infect Dis:ciaa121.
51. Karvanen M, Malmberg C, Lagerbäck P, Friberg LE, Cars O. 2017. Colistin is extensively lost during standard *in vitro* experimental conditions. Antimicrob Agents Chemother 61:1–21.
52. Huys G, Cnockaert M, Janda JM, Swings J. 2003. *Escherichia albertii* sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. Int J Syst Evol Microbiol 53:807–810.
53. Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. 2010. Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a Clinical Microbiology Laboratory. J Clin Microbiol 48:1549–1554.
54. Rodrigues C, Passet V, Rakotondrasoa A, Diallo TA, Criscuolo A, Brisse S. 2019. Description of *Klebsiella africanensis* sp. nov., *Klebsiella variicola* subsp. *tropicalensis* subsp. nov. and *Klebsiella variicola* subsp. *variicola* subsp. nov. Res Microbiol 170:165–170.
55. Antoniadou A, Kontopidou F, Poulakou G, Koratzanis E, Galani I, Papadomichelakis E, Kopterides P, Souli M, Armaganidis A, Giamarellou H. 2007. Colistin-resistant isolates of *Klebsiella pneumoniae* emerging in intensive care unit patients: first report of a multiclonal cluster. J Antimicrob Chemother 59:786–790.
56. Mammina C, Bonura C, Di Bernardo F, Aleo A, Fasciana T, Sodano C, Saporito M a, Verde MS, Tetamo R, Palma DM. 2012. Ongoing spread of colistin-resistant *Klebsiella pneumoniae* in different wards of an acute general hospital, Italy, June to December 2011. Eurosurveillance 17:pii=20248.
57. Parisi SG, Bartolini A, Santacatterina E, Castellani E, Ghirardo R, Berto A, Franchin E, Menegotto N, De Canale E, Tommasini T, Rinaldi R, Basso M, Stefani S, Palù G. 2015. Prevalence of *Klebsiella pneumoniae* strains producing carbapenemases and increase of resistance to colistin in an Italian teaching hospital from January 2012 to December 2014. BMC Infect Dis 15:244.
58. Rossi F, Girardello R, Cury AP, Di Gioia TSR, Almeida JN de, Duarte AJ da S. 2017. Emergence of colistin resistance in the largest university hospital complex of São Paulo, Brazil, over five years. Brazilian J Infect

Dis 21:98–101.
59. Falagas ME, Kasiakou SK. 2006. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit care 10:R27.
60. Nation RL, Li J, Cars O, Couet W, Dudley MN, Kaye KS, Mouton JW, Paterson DL, Tam VH, Theuretzbacher U, Tsuji BT, Turnidge JD. 2015. Framework for optimisation of the clinical use of colistin and polymyxin B: the Prato polymyxin consensus. Lancet Infect Dis 15:225–234.
61. Korucu B, Unal I, Pekcan M, Inkaya AC, Yeter H, Cetinkaya MA, Kaymaz FF, Unal S, Akova M, Erdem Y. 2019. Ultrastructural evaluation of urine alkalinization versus hydration on colistin-induced nephrotoxicity. Hum Exp Toxicol 38:1366–1377.
62. Li J, Nation RL, Kaye KS. 2019. Polymyxin antibiotics: from laboratory bench to bedside.
63. World Health Organization. 2019. 2019 Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline.
64. World Health Organization. 2019. Antibacterial agents in preclinical development: an open access database.
65. Fabijan AP, Lin RC, Ho J, Maddocks S, Zakour NL, Iredell JR, Westmead Bacteriophage Therapy Team. 2020. Safety of bacteriophage therapy in severe *Staphylococcus aureus* infection. Nat Microbiol 5:465–472.
66. Jault P, Leclerc T, Jennes S, Pirnay JP, Que YA, Resch G, Rousseau AF, Ravat F, Carsin H, Le Floch R, Schaal JV, Soler C, Fevre C, Arnaud I, Bretaudeau L, Gabard J. 2019. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. Lancet Infect Dis 19:35–45.
67. Zucca M, Savoia D. 2010. The post-antibiotic era: promising developments in the therapy of infectious diseases. Int J Biomed Sci 6:77–86.
68. Heesterbeek DAC, Angelier ML, Harrison RA, Rooijakkers SHM. 2018. Complement and bacterial infections from molecular mechanisms to therapeutic applications. J Innate Immun 10:455–464.
69. Son SJ, Huang R, Squire CJ, Leung IKH. 2019. MCR-1: a promising target for structure-based design of inhibitors to tackle polymyxin resistance. Drug Discov Today 24:206–216.
70. Xu Y, Wei W, Lei S, Lin J, Srinivas S, Feng Y. 2018. An evolutionarily conserved mechanism for intrinsic and transferable polymyxin resistance. mBio 9:e02317-17.
71. Harris TL, Worthington RJ, Hittle LE, Zurawski D V, Ernst RK, Melander C. 2014. Small molecule downregulation of PmrAB reverses lipid A modification and breaks colistin resistance. ACS Chem Biol 9:122–127.
72. D'Agata EMC, Horn MA, Ruan S, Webb GF, Wares JR. 2012. Efficacy of infection control interventions in reducing the spread of multidrug-resistant organisms in the hospital setting. PLoS One 7:e30170.
73. Rex JH, Outterson K. 2016. Antibiotic reimbursement in a model delinked from sales: a benchmark-based worldwide approach. Lancet Infect Dis 16:500–505.
74. European Commission. 2011. Action plan against the rising threats form antimicrobial resistance.
75. Government of the United States of America. 2015. National action plan for combating antibiotic-resistant bacteria.
76. World Health Organization. 2015. Global action plan on antimicrobial resistance.
77. Walsh TR, Wu Y. 2016. China bans colistin as a feed additive for animals. Lancet Infect Dis 16:1102–1103.
78. European Medicines Agency. 2016. Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health.

Chapter

Closing pages

Nederlandse Samenvatting

Introductie

Antibiotica spelen een cruciale rol in de moderne gezondheidszorg in de preventie en behandeling van bacteriële infecties. Het toenemend voorkomen van resistentie tegen meerdere antibiotica (multiresistentie) in Gram-negatieve bacteriën die opportunistische infecties veroorzaken is in toenemende mate een probleem voor behandelingen. Bekende Gram-negatieve bacteriën die opportunistische infecties veroorzaken zijn *Escherichia coli* en *Klebsiella pneumoniae*. *E. coli* en *K. pneumoniae* zijn beide soorten in de familie Enterobacteriaceae. Indien *E. coli* en *K. pneumoniae* resistentie hebben ontwikkeld tegen antibioticagroepen zoals fluoroquinolonen, aminoglycosiden, en derde-generatie cefalosporines zijn er nog weinig middelen beschikbaar om infecties te bestrijden. In het licht van deze toenemende multiresistentie heeft colistine een wederopleving meegemaakt. Colistine werd veelvuldig gebruikt halverwege de 20^{e} eeuw, maar omdat colistine bijwerkingen heeft op de nieren en het zenuwstelsel, raakte het in onbruik nadat er antibiotica waren ontwikkeld met minder bijwerkingen. Nu wordt colistine weer gebruikt als laatste redmiddel voor de behandeling van multiresistente Gram-negatieve bacteriën. Colistine is een antibioticum uit de polymyxine klasse, en werkt specifiek op Gram-negatieve bacteriën doordat het bind aan een component van de buitenmembraan van Gram-negatieve bacteriën; het lipide A gedeelte van lipopolysachariden (LPS). De LPS moleculen zijn alleen aanwezig in de membranen van Gram-negatieve bacteriën. Deze binding vindt plaats op basis van elektrostatische interacties tussen het positief geladen colistine molecuul, en de negatief geladen fosfaatgroepen van lipide A. Nadat colistine zich heeft gebonden aan de lipide A van de LPS moleculen, kan het zich in de buitenmembraan van de bacterie steken. Dit proces herhaalt zich vervolgens ook in de binnenmembraan van de bacterie. Het insteken van de colistine in de membranen zorgt ervoor dat deze zijn integriteit verliezen. Het verlies van integriteit zorgt ervoor dat de bacterie doodgaat.

Het doel van dit proefschrift

Doordat colistine in onbruik was geraakt, is er weinig onderzoek gedaan naar de mechanismen die kunnen leiden tot resistentie. In dit proefschrift hebben wij onderzoek gedaan naar de mutaties die kunnen leiden tot colistine resistentie in *E. coli* en *K. pneumoniae*. Ook hebben we gekeken naar de evolutionaire wegen die deze bacteriën kunnen gebruiken om resistent te worden tegen colistine. We

onderzochten ook de impact van de ontwikkeling van colistine resistentie op de virulentie die een bacterie heeft. Als laatste onderzochten we de verspreiding van colistine-resistente bacteriën tussen patiënten. Als er meer bekend is over de oorzaken en gevolgen van het ontwikkelen van colistine resistentie, kunnen daar mogelijk nieuwe therapieën op worden gebaseerd.

Resistentiemechanismen in klinische Enterobacteriaceae isolaten

Enterobacteriaceae zijn welbekend om hun vermogen om infecties te veroorzaken. Zowel in gezonde, als immuungecompromitteerde patiënten, kunnen zij urineweg-, long-, en bloedbaaninfecties veroorzaken. Indien multiresistente bacteriën in een bloedbaan infectie resistent zouden zijn tegen colistine, zou dit de behandeling van deze infectie met een dergelijke multiresistente stam nog moeilijker maken. In **Hoofdstuk 2** hebben wij onderzocht hoeveel colistine-resistente *E. coli* stammen er in de laatste tien jaar in het Universitair Medisch Centrum (UMC) Utrecht zijn geïsoleerd uit bloedbaan infecties. Ook onderzochten wij de mutaties die hebben geleid tot de colistine-resistentie. Door retrospectieve screening vonden we dat er van de 1140 *E. coli* stammen geïsoleerd uit bloedbaan infecties die zijn opgeslagen, er maar tien resistent waren tegen colistine. Van de tien patiënten van wie deze stammen zijn geïsoleerd, waren er maar drie behandeld met colistine in de periode voorafgaand aan de isolatie. Als we de lipide A van de LPS van deze stammen analyseren doormiddel van massaspectrometrie, zien we dat deze stammen een gemodificeerd lipide A hebben. De lipide A van al deze stammen is gemodificeerd door de toevoeging van een positief geladen fosfaatethanolamine groep aan een van de twee negatieve fosfaatgroepen van lipide A. Door deze modificatie kan colistine zich minder goed binden aan lipide A. Deze modificatie wordt aangebracht door de fosfaatethanolamine-transferase EptA. Het gen wat EptA codeert wordt transcriptioneel gecontroleerd door het BasRS signaleringssysteem. Door de chromosomale genoom sequentie van deze colistine-resistente stammen te vergelijken met die van nauw verwante colistine-gevoelige stammen, en vervolgens het maken van chromosomale mutanten, vonden we mutaties in de genen van BasR en BasS in vier stammen die colistine resistentie veroorzaakten. De colistine-resistente stammen zijn niet nauw verwant aan elkaar; er was dus geen uitbraak van colistine-resistente *E. coli* stammen. Uit de analyse van het genoom van deze stammen bleek dat één stam het *mcr-1.1*-gen bij zich droeg. Deze was gelokaliseerd op een plasmide wat wereldwijd is verspreid.

Colistine wordt niet alleen gebruikt in de behandeling van infecties met Gram-negatieve bacteriën, het wordt ook gebruikt om infecties te voorkomen.

Patiënten die op een intensive care (IC) worden opgenomen worden veelal preventief behandeld met een behandeling die selectieve decontaminatie van het digestieve stelsel (SDD) heet. De SDD behandeling is erop gericht om de bacteriën in het digestieve stelsel te doden zodat zij geen opportunistische infecties kunnen veroorzaken in deze kwetsbare patiëntenpopulatie. Deze behandeling bestaat uit de orale toediening van een mengsel van colistine, tobramycine, en amfotericine B, en het intraveneus toedienen van derde-generatie cefalosporine die via de gal wordt uitgescheiden. Deze behandeling is effectief in het terugdringen van morbiditeit en mortaliteit van IC-patiënten door het terugdringen van infecties. De SDD behandeling is echter alleen effectief bevonden in landen waar een lage prevalentie van antibioticaresistentie wordt gevonden. Het ontstaan van resistentie tegen een van de antibiotica die wordt gebruikt zou dus de effectiviteit van SDD kunnen bedreigen. Vanwege het belang van het ontstaan van resistentie voor de effectiviteit van SDD, moet er tijdens de behandeling worden gesurveilleerd voor resistente stammen. In **Hoofdstuk 3** en **Hoofdstuk 4** maken wij gebruik van de colistine-resistente Enterobacteriaceae stammen die zijn gevonden tijdens deze routine surveillance. Wij analyseerde de genomen van deze bacteriën voor de mechanismen van colistine resistentie die kunnen optreden onder invloed van het gebruik van SDD in de IC.

In **Hoofdstuk 3** onderzoeken we de colistine-resistente stammen die waren verkregen van vijf patiënten opgenomen op de IC in het UMC Utrecht. In totaal waren er dertien colistine-resistente Enterobacteriaceae stammen geïsoleerd; negen *E. coli* stammen, drie *K. aerogenes* stammen, en een *Enterobacter asburiae* stam. Zeven van de negen *E. coli* stammen waren uit één patiënt geïsoleerd en waren klonaal aan elkaar gerelateerd. In deze klonale *E. coli* stammen observeerde wij resistentie-genen tegen tobramycine en derde-generatie cefalosporinen. In deze zeven *E. coli* isolaten vinden we dat de colistine resistentie wordt veroorzaakt door twee verschillende mutaties in de genen die de BasR (aangedaan in drie van de zeven isolaten) en BasS (aangedaan in vier isolaten) eiwitten coderen. Het ontstaan van twee verschillende mutaties die bijdragen aan colistine resistentie indiceert dat er een colistine-gevoelige stam aanwezig was, die onder druk van colistine via verschillende mutaties resistentie heeft ontwikkeld. Door deze resistentie wordt deze stam niet gedood door de SDD behandeling, en kan de stam het digestieve stelsel van deze patiënt koloniseren. Deze observatie zou een waarschuwing moeten zijn dat het gebruik van SDD kan leiden tot het ontstaan van multiresistente bacteriën, die ook resistent zijn tegen colistine.

De drie *K. aerogenes* (afkomstig uit een patiënt), *E. asburiae*, en twee *E. coli* stammen (uit twee patiënten afkomstig) komen uit 4 individuele andere patiënten. In het geval van de *K. aerogenes* stammen, hadden we ook de beschikking over een

colistine-gevoelige *K. aerogenes* stam van dezelfde patiënt, die klonaal was met de colistine-resistente stammen. Door deze stammen te vergelijken vinden we dat er een mutatie in BasR had plaatsgevonden (PmrA genaamd in *Klebsiella*). Deze mutatie had dus plaatsgevonden in de patiënt, en heeft geleid tot het ontstaan van colistine-resistentie. Van de *E. asburiae* stam bevinden wij dat deze resistent is tegen hoge concentraties colistine. In een van de *E. coli* stammen vinden we een, wederom wereldwijd verspreid, plasmide met een *mcr-1.1* gen. De laatste *E. coli* stam lijkt resistent te zijn geworden tegen colistine door mutatie in het gen van BasS.

Gelijk aan de bevindingen dat een *E. coli* stam die resistent is tegen derde-generatie cefalosporines en aminoglycosiden succesvol kan zijn in het koloniseren van het digestieve stelsel van een patiënt, bevinden we in **Hoofdstuk 4** dat multiresistente *K. pneumoniae* hetzelfde kan doen. We vinden stammen die waren geïsoleerd tijdens een uitbraak op een IC van *K. pneumoniae* die resistent was tegen aminoglycosiden en derde generatie cefalosporines, allemaal colistine-resistentie hebben ontwikkeld via unieke mutaties. De introductie van SDD op deze IC leidde niet tot een vermindering van de uitbraak, maar juist tot het ontwikkelen van colistine resistentie in deze stammen door het ontstaan van mutaties in verschillende genen. De vinding van verschillende mutaties in verschillende genen benadrukt de flexibiliteit die *K. pneumoniae* lijkt te hebben in het ontwikkelen van colistine resistentie.

Gecontroleerde evolutie van colistine resistentie in K. pneumoniae in het laboratorium

In **Hoofdstuk 2**, **Hoofdstuk 3**, en **Hoofdstuk 4** onderzochten we de mutaties die hebben geleiden tot colistine resistentie in Enterobacteriaceae isolaten verkregen van patiënten. Deze isolaten waren geïsoleerd uit bloedbaan infecties of van patiënten op de IC die de SDD behandeling kregen. Ondanks dat we wel de mutaties kunnen ontdekken die in deze isolaten hadden geleid tot colistine resistentie, konden we niet de evolutionaire mechanismen en paden opmaken die de stammen hadden bewandeld om resistent te worden. Het gaat in deze processen om de snelheid en de selectiviteit van het ontstaan van de mutaties die tot resistentie leiden. Om de exacte evolutionaire processen te kunnen ontrafelen die er plaatsvinden als een stam resistentie tegen colistine ontwikkelt, hebben we in **Hoofdstuk 5** een evolutie experiment in het lab uitgevoerd met vier klinisch relevante, colistine-gevoelige *K. pneumoniae* complex stammen. We hebben deze vier stammen een aantal dagen gekweekt in vloeibare culturen, waarin we elke dag de concentratie colistine verhoogde, waardoor wij selectieve

druk hebben uitgeoefend op het ontstaan van colistine resistentie. Wij zagen in deze culturen dat er een snelle ontwikkeling was van colistine resistentie. Door de genoom sequenties van alle bacteriën in de culturen te bepalen, konden we zien hoe snel een bacterie met een spontane mutatie die leidt tot colistine resistentie, andere bacteriën zonder deze mutatie eruit concurreert onder invloed van de toenemende colistine concentraties. Door deze analyses vinden we dat indien een bacterie een mutatie ontwikkelt die leidt tot colistine resistentie, hij zeer snel de plek van andere bacteriën zonder deze mutatie kan overnemen in de cultuur. We vinden dat niet alleen het veranderen van één of enkele basen in genen die te doen hebben met LPS biosynthese of capsule structuren, kan leiden tot resistentie, maar ook het integreren van grotere insertie sequentie (IS) elementen, die zichzelf kunnen verplaatsen in het genoom. De integratie van deze IS elementen kan tot de disruptie van genen leiden. Integratie van een IS element kan ook leiden tot de waarschijnlijke verandering van expressie, door in de controle-regio's van genen te integreren.

Omdat we een gecontroleerd laboratorium evolutie experiment hebben uitgevoerd, hebben we voor deze *K. pneumoniae* complex stammen zowel colistine-gevoelige, als colistine-resistente stammen beschikbaar. Hierdoor konden we de veranderingen in de *K. pneumoniae* complex stammen goed in kaart brengen. Door massaspectrometrie vonden we dat de lipide A van de colistine-resistente *K. pneumoniae* complex stammen, in vergelijking met de lipide A van de colistine-gevoelige stammen is gemodificeerd met 4-amino-4-deoxy-L-arabinose, hydroxyl-groepen, of palmitaat-groepen. We hebben de beschikbaarheid van de colistine-gevoelige en –resistente stammen ook gebruikt om de invloed van colistine-resistentie op de mate van ziekmakendheid (virulentie) van deze bacteriën te bestuderen. Omdat het LPS (en dus het lipide A) molecuul een erg belangrijke factor is voor de virulentie van Gram-negatieve bacteriën, kunnen veranderingen in het lipide A leiden tot een verandering van de virulentie. We vonden een variabel effect op deze virulentie. Drie stammen werden minder gevoelig voor een menselijk antimicrobieel eiwit, uitgescheiden door fagocyten, door het ontwikkelen van colistine resistentie. Twee van de vier stammen werden gevoeliger voor antimicrobiële eiwitten die zich in ons bloed bevinden. Door het bestuderen van de virulentie in een nematode model, vonden we dat twee van de vier stammen meer virulent werden als ze resistent tegen colistine zijn. Al met al vinden we dat *K. pneumoniae* complex stammen verschillende evolutionaire paden kan bewandelen om resistent te worden tegen colistine, en dat deze resistentie zich snel kan ontwikkelen.

Ter conclusie

Door het onderzoek beschreven in dit proefschrift begrijpen we meer over hoe klinisch relevante Enterobacteriaceae stammen resistentie tegen colistine kunnen ontwikkelen. In de bestudeerde stammen zien we een grote verscheidenheid aan mutaties die kunnen leiden tot colistine resistentie. Desondanks zien we dat deze mutaties veelal in dezelfde genen plaatsvinden. Deze observaties leiden wellicht tot de identificatie van nieuwe eiwitten die kunnen worden beïnvloed met medicijnen om de ontwikkeling van colistine-resistentie onmogelijk te maken. Ook zien we dat het profylactisch inzetten van colistine, kan leiden tot de selectie van multiresistente bacteriën in de IC. Ook hebben we gekeken naar de gevolgen van colistine-resistentie voor de virulentie van de bacterie.

Ondanks dat er een grotere nadruk op het juiste gebruik van antibiotica in de humane als veterinaire geneeskunde is gelegd in de afgelopen jaren, blijft de dreiging van moeilijk te behandelen multiresistente stammen bestaan. Daarom is er nog steeds meer onderzoek nodig naar de mechanismen en verspreiding van antibiotica resistentie (waaronder colistine). Nieuw onderzoek zou tot de ontdekking van tot nog toe onbekende resistentie mechanismen kunnen leiden, en de identificatie van nieuwe doelen in bacteriën zodat de ontwikkeling van colistine resistentie een halt kan worden toegeroepen.

Acknowledgements

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List of publications

Related to this thesis

Janssen AB, van Hout D, Bonten MJM, Willems RJL, van Schaik W. 2020. Microevolution of acquired colistin resistance in Enterobacteriaceae from ICU patients receiving selective decontamination of the digestive tract. Journal of Antimicrobial Chemotherapy: dkaa305

Janssen AB, Doorduijn DJ, Mills G, Rogers MRC, Bonten MJM, Rooijakkers RHM, Willems RJL, Bengoechea JA, van Schaik W. 2020. *In vitro* evolution of colistin resistance in the *Klebsiella pneumoniae* complex follows multiple evolutionary trajectories with variable effects on fitness and virulence characteristics. bioRxiv: 2020.05.24.112334

Janssen AB, Bartholomew TL, Marciszewska NP, Bonten MJM, Willems RJL, Bengoechea JA, van Schaik W. 2020. Nonclonal emergence of colistin resistance associated with mutations in the BasRS two-component system in *Escherichia coli* bloodstream isolates. mSphere 5:e00143-20

Halaby T*, Küçükköse E*, **Janssen AB**, Rogers MRC, Doorduijn DJ, van der Zanden AGM, Al Naiemi N, Vandenbroucke-Grauls CMJE, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. Antimicrobial Agents and Chemotherapy 60:6837-6843.

Other publications

ten Doesschate T*, Moorlag SJCFM*, van der Vaart TW, Taks E, Debisarun P, ten Oever J, Bleeker-Rovers CP, Bruijning-Verhagen P, Lalmohamed A, ter Heine R, van Crevel R, van de Wijgert J, **Janssen AB**, Bonten MJM, van Werkhoven CH*, Netea MG*, on behalf of the BCG-CORONA studyteam. 2020. Two randomized controlled trials of Bacillus Calmette-Guérin Vaccination to reduce absenteeism among health care workers and hospital admission by elderly persons during the COVID-19 pandemic: a structured summary of the study protocols for two randomised controlled trials. Trials 21:555.

van Hout D, **Janssen AB**, Rentenaar RJ, Vlooswijk JPM, Boel CHE, Bonten MJM. 2019. The added value of the selective SuperPolymyxin™ medium in detecting rectal carriage of Gram-negative bacteria with acquired colistin resistance in intensive care unit patients receiving selective digestive decontamination. European Journal of Clinical Microbiology & Infectious Diseases 39:265-271

Vercoulen Y*, Konda Y*, Iwig JS*, **Janssen AB**, White KA, Amini M, Barber DL, Kuriyan J, Roose JP. 2017. A Histidine pH sensor regulates activation of the Ras-specific guanine nucleotide exchange factor RasGRP1. eLife 6:e29002.

de Gruijl TD, **Janssen AB**, van Beusechem VW. 2015. Arming oncolytic viruses to leverage antitumor immunity. Expert Opinion on Biological Therapy 15:959-971.

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About the author

Absalom (Axel) Benjamin Janssen was born on October 31st 1991 in Nijmegen, the Netherlands. In 2010, he graduated from secondary education with a gymnasium degree from the Stedelijk Gymnasium Nijmegen, in Nijmegen, the Netherlands.

Later that year, he started his Bachelor of Science degree in Biomedical Sciences at Utrecht University, in Utrecht, the Netherlands. For his bachelor's thesis, he performed an internship on colistin resistance under supervision of prof. dr. ir. Willem van Schaik, in the Department of Medical Microbiology at the University Medical Center Utrecht (UMC Utrecht), in Utrecht, the Netherlands.

After graduating from the bachelor Biomedical Sciences in 2013 with distinction, he started his Master of Science degree in Infection and Immunity at the same university later that year. For this master's degree, Axel performed a nine-month internship on colistin resistance in Gram-negative bacteria, again under supervision of prof. dr. ir. Willem van Schaik, at the Department of Medical Microbiology in the UMC Utrecht. For his writing assignment, Axel wrote a literature review on the genetic adaptation of oncolytic viruses, under supervision of prof. dr. Victor W. van Beusechem, and prof. dr. Tanja D. de Gruijl at the VU University Medical Center (now Amsterdam UMC, location VUmc), in Amsterdam, the Netherlands. After, Axel performed an eight-month internship on the effects of intracellular pH on Ras activation under supervision of dr. Yvonne Vercoulen, in the group of prof. dr. Jeroen P. Roose, located on the Parnassus campus of the University of California, San Franscisco. Axel graduated from the Infection and Immunity master in December 2015.

In January 2016, Axel started his PhD degree at the UMC Utrecht under supervision of prof. dr. ir. Willem van Schaik, continuing the work from his nine-month internship. The results of this work have been presented at multiple (inter)national conferences, submitted for publication in peer-reviewed international scientific journals, and are collected in this thesis.