Nonclonal Emergence of Colistin Resistance Associated with Mutations in the BasRS Two-Component System in *Escherichia coli* Bloodstream Isolates

Axel B. Janssen,[a] Toby L. Bartholomew,[b] Natalia P. Marciszewska,[a] Marc J. M. Bonten,[a] Rob J. L. Willems,[a] Jose A. Bengoechea,[b] Willem van Schaik[a,c]

[a]Department of Medical Microbiology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[b]Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom
[c]Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

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 *Escherichia albertii* strain. These were the only colistin-resistant strains of 1,140 bloodstream *Escherichia* isolates collected in a tertiary hospital over a 10-year period (2006 to 2015). Core-genome phylogenetic analysis showed that each patient was colonized 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 characterize the mechanisms through which *Escherichia 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.

KEYWORDS *Escherichia coli*, antibiotic resistance, colistin, two-component regulatory systems, whole-genome sequencing

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 nonribosomal 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 catalyze 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 that in 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 controlled not only by MgrB but also by the small RNA (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 leads 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 ionization–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.

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 3 months before the isolation of the colistin-resistant isolate, and if applicable, route of administration.
[b]The MIC values represent the medians from three independent replicate experiments performed in triplicates.

RESULTS

Low prevalence of colistin resistance in invasive *Escherichia* bloodstream isolates. A total of 1,140 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 10 of the 11 remaining isolates, colistin resistance, defined as an MIC of >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 3 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 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 (see Table S1 in the supplemental material). Based on a core genome alignment of 874 kbp, we did not observe direct transmission of colistin-resistant strains between patients (Fig. 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 (Fig. 1A and B) (3, 34). This indicates that the ST131 strains in this study have independently acquired colistin resistance. Strain A2361 clustered among *Escherichia albertii* (Fig. 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) (Fig. 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
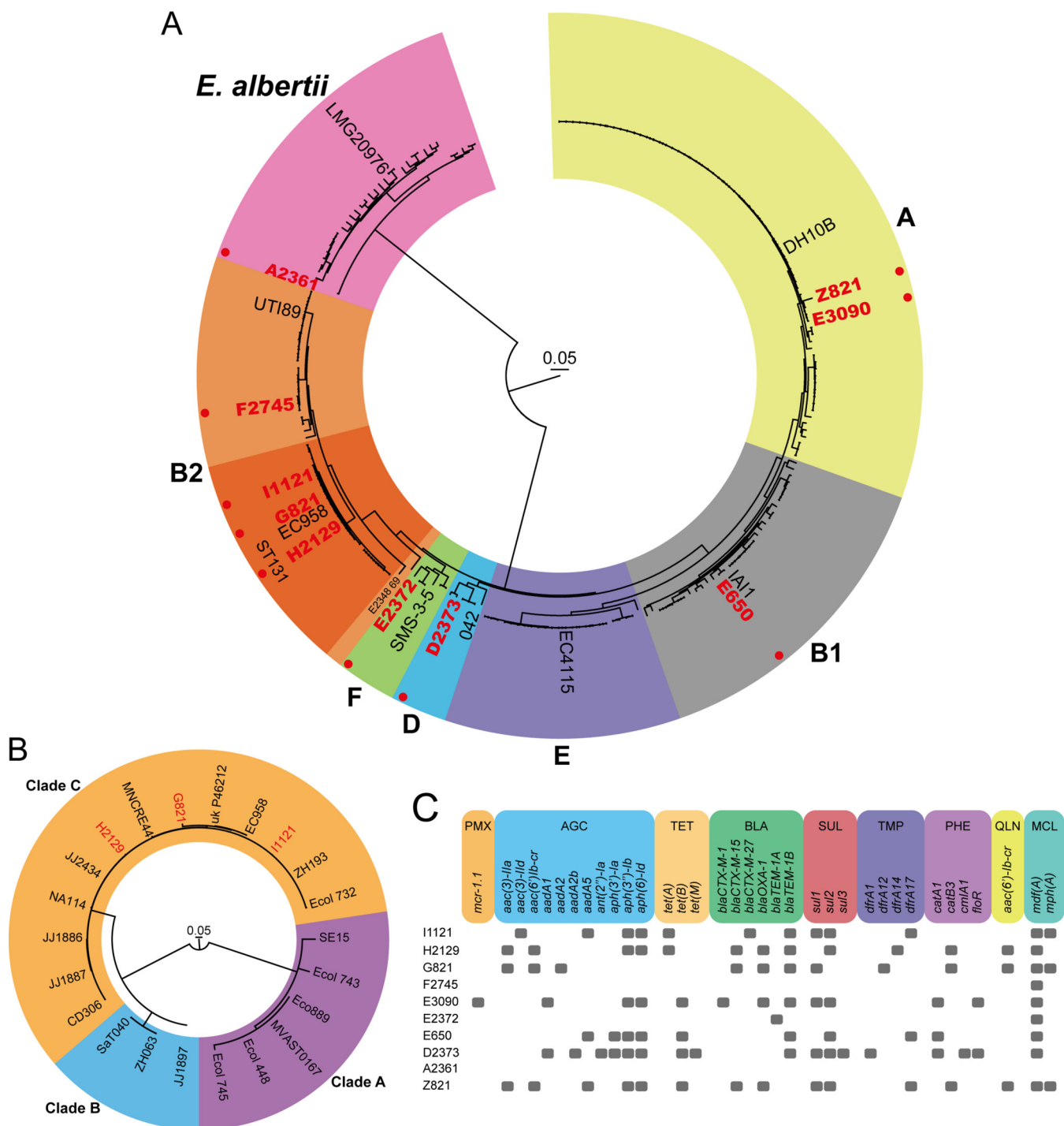
FIG 1 Colistin-resistant strains are not clonally related and carry diverse acquired antibiotic resistance genes. (A) The phylogenetic tree represents the core-genome alignment (874 kbp) of the colistin-resistant strains and 210 publicly available *E. coli* and *E. albertii* genome sequences. One representative 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 colored backgrounds. Phylogroup A is yellow; B1, gray; 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 follows: PMX, polymyxin resistance; AGC, aminoglycoside resistance; TET, tetracycline resistance; BLA, beta-lactam resistance; SUL, sulfonamide resistance; TMP, trimethoprim resistance; PHE, phenicol resistance; QLN, quinolone resistance; MCL, macrolide resistance.

(GenBank accession MK172815.1) harbored 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 (Fig. 1C), reflecting the nonclonal 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. Strains F2745 and E2372 carried only one and two resistance genes, respectively, while the *E. albertii* strain A2361 did not possess any acquired resistance genes.

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 1,797 (Fig. 2a), corresponding to the canonical unmodified *E. coli* hexa-acylated lipid A (Fig. 2b). Colistin-resistant strains showed additional lipid A species at *m/z* 1,921, 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. The species at *m/z* 2,036 indicated the addition of palmitate (*m/z* 239) to the hexa-acylated species at *m/z* 1,797, whereas the species at *m/z* 2,160 was consistent with the addition of palmitate to the hexa-acylated lipid A species containing phosphoethanolamine (*m/z* 1,910).

The *E. albertii* strain A2361 produced lipid A distinct from that by *E. coli*. The species at *m/z* 1,825 is likely to represent a hexa-acylated species corresponding to two glucosamines, two phosphates, four 3-OH-C_{14}, and two C_{14} (Fig. 2c). The species at *m/z* 1,948 is consistent with the addition of phosphoethanolamine to the hexa-acylated species, with a further addition of palmitate to produce lipid A species at *m/z* 2,187. Species at *m/z* 1,868 and *m/z* 2,107 could correspond to the loss of the second phosphate group, compared to those at *m/z* 1,948 and *m/z* 2,187.

Identification of mutations in *basRS* as candidate mutations involved in colistin resistance. Because chromosomal mutations in *basRS*, but not in other regulatory systems, were previously 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 (Fig. 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*Tn7 site in the BW25113-derived Δ*basRS* strain BW27848 using the Tn7 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 the phosphoethanolamine transferase EptA, we constructed sequences that consisted of the fused sequences of the promoter 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::Tn7-empty strain, with observed MIC values ≥16-fold higher than that of the BW27848::Tn7-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, and 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
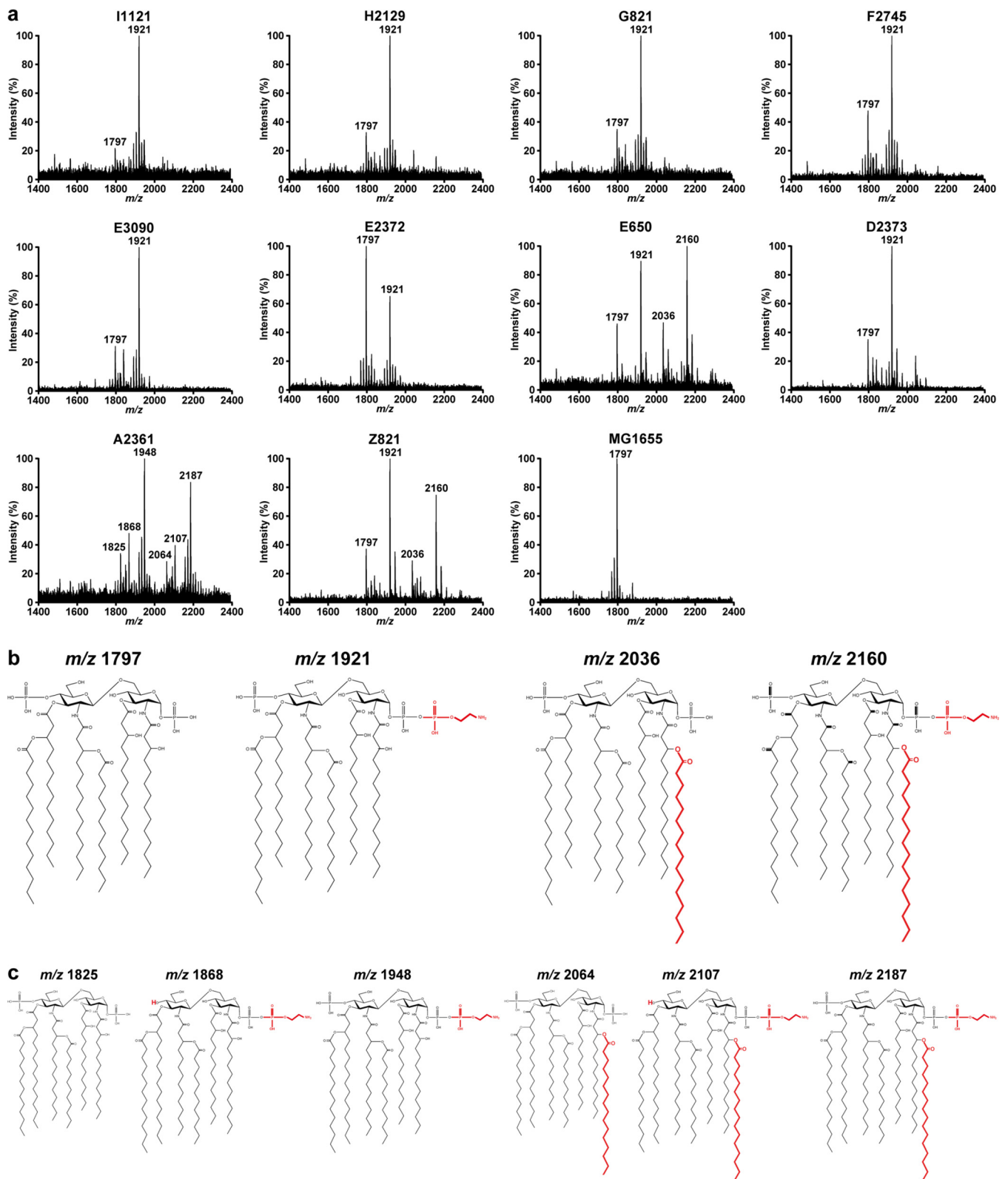
FIG 2 MALDI-TOF spectra of lipid A from colistin-resistant nosocomial *Escherichia* strains. (a) Negative ion MALDI-TOF mass spectrometry spectra of lipid A purified from colistin-resistant strains and colistin-susceptible MG1655. Data represent the mass to charge (*m/z*) ratios of each lipid A species 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.

**TABLE 2** Colistin MICs of strains generated in this study[a]

Strain	Colistin MIC (μg/ml)[b]
BW25113	0.25
BW27848	0.125
BW25113::Tn7 empty	0.25
BW27848::Tn7 empty	0.125
BW27848::Tn7 BW25113	0.125
BW27848::Tn7 I1121	2
BW27848::Tn7 I1121m	0.25
BW27848::Tn7 H2129	4
BW27848::Tn7 H2129m	0.25
BW27848::Tn7 G821	4
BW27848::Tn7 G821m	0.25
BW27848::Tn7 F2745	0.25
BW27848::Tn7 E3090	0.125
BW27848::Tn7 E2372	0.25
BW27848::Tn7 D2373	0.5
BW27848::Tn7 A2361	0.125
BW27848::Tn7 Z821	2
BW27848::Tn7 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*Tn7 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 medians from three independent replicate experiments performed in triplicates.

resistance in BW27848. To identify the mutations in the *basRS* alleles of these strains that contribute to resistance, we compared the *basRS* sequences of those strains causing resistance to the phylogenetically most closely related publicly available *E. coli* genome sequences used in the construction of Fig. 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: an L10R substitution in BasS in I1121, a G53S substitution in BasR in H2192, the duplication of the HAMP-domain in BasS in G821, and an A159P substitution in BasS in Z821 (Fig. 3). As expected, in the *mcr-1.1*-positive strain E3090, no mutations in *basRS* were identified.

We hypothesized 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 Z82, and their closest match in the set of 178 publicly available *E. coli* genome sequences (Fig. 3) were causal to the development of colistin resistance, the identified mutations were reversed through site-directed inverse PCR mutagenesis to match the
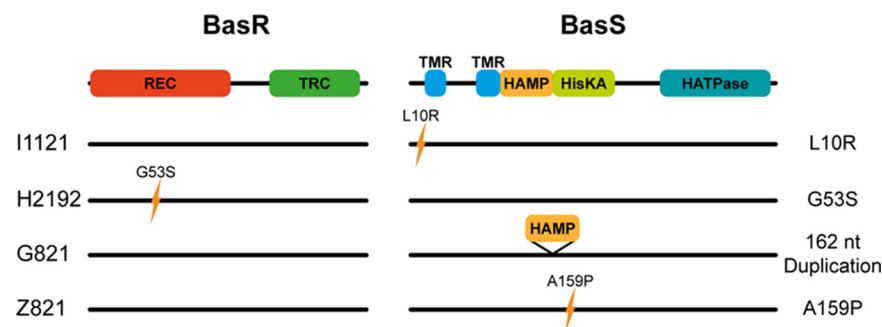
FIG 3 Conservation and prediction of functional effects of mutations in *basRS*. Comparison of the *basRS* sequences of colistin-resistant strains and publicly available genome sequences led to the identification of mutations in *basRS* that could have a role in colistin resistance. Domains of BasR and BasS were predicted using SMART (55). The domains are as follows: 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.

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*.

DISCUSSION

In the present study, we set out to characterize 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 was 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 was previously 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 hypothesize 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 an A159V substitution was found in an *in vitro* evolution study in which *E. coli* was evolved toward 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 such as colistin. While the discovery of the *mcr* genes has 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 tryptic soy agar (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 mg/liter; Sigma-Aldrich, Zwijndrecht, The Netherlands) and ampicillin (100 mg/liter; Sigma-Aldrich) were used. Colistin sulfate 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 gels using GeneJET Gel Extraction and DNA Cleanup Micro kits (Thermo Fisher Scientific).

Determination of MIC. MICs to 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) (67), using BBL Mueller-Hinton II (cation-adjusted) broth (MHCAB; Becton, Dickinson), untreated Nunc 96-well round-bottom polystyrene plates (Thermo Scientific), and Breathe-Easy sealing membranes (Sigma-Aldrich). The breakpoint value of an MIC of >2 μg/ml for colistin resistance in *E. coli* was obtained from EUCAST (http://www.eucast.org/clinical_breakpoints/).

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 two-dimensional (2D) long-read sequencing was performed using the SQK-LSK208 kit (Oxford Nanopore Technologies, Oxford, United Kingdom), according to the manufacturer's instructions, with G-tube (Covaris, Woburn, MA, USA) shearing of 1 μg chromosomal DNA for 2 × 120 s at 1,500 × *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, 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 cutoffs for final assemblies for a minimum contig/scaffold size of 500 bp and minimum contig/scaffold average nucleotide coverage at 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). Multilocus sequence typing (MLST) 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 (see Table S1 in the supplemental material) using ParSNP v1.2 (57). MEGA6 was used to midpoint root and visualize the phylogenetic tree (58). We identified whether nonsynonymous 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 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 equal volumes 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 Tn7 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 promoter region and the *basRS* amplicon, with high fidelity Phusion Green Hot Start II DNA polymerase (Thermo Fisher Scientific) using strain-specific primers (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, 1,800 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 Tn7 transposon at the *att*Tn7 site by PCR (primers listed in 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, MA, USA). 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 have been deposited in the European Nucleotide Archive (accession number PRJEB27030).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

REFERENCES

1. Kaper JB, Nataro JP, Mobley H. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140. https://doi.org/10.1038/nrmicro818.

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. https://doi.org/10.1101/gr.216606.116.

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. https://doi.org/10.1128/mBio.00644-19.

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 U S A 115:E3463–E3470. https://doi.org/10.1073/pnas.1717295115.

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. https://doi.org/10.1592/phco.30.12.1279.

6. Nation RL, Li J. 2009. Colistin in the 21st century. Curr Opin Infect Dis 22:535–543. https://doi.org/10.1097/QCO.0b013e328332e672.

7. Domingues MM, Inácio RG, Raimundo JM, Martins M, Castanho M, Santos NC. 2012. Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. Biopolymers 98: 338–344. https://doi.org/10.1002/bip.22095.

8. Landman D, Georgescu C, Martin DA, Quale J. 2008. Polymyxins revisited. Clin Microbiol Rev 21:449–465. https://doi.org/10.1128/CMR.00006-08.

9. Velkov T, Thompson PE, Nation RL, Li J. 2010. Structure-activity relationships of polymyxin antibiotics. J Med Chem 53:1898–1916. https://doi.org/10.1021/jm900999h.

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 479618. https://doi.org/10.1101/479618.

11. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, 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. https://doi.org/10.1016/S1473-3099(15)00424-7.

12. Halaby T, Kucukkose E, Janssen AB, Rogers MRC, Doorduijn DJ, van der Zanden AGM, Al Naiemi N, Vandenbroucke-Grauls C, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneu-*

moniae during a nosocomial outbreak. Antimicrob Agents Chemother 60:6837–6843. https://doi.org/10.1128/AAC.01344-16.

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. https://doi.org/10.1186/s13099-017-0227-4.

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. https://doi.org/10.1016/j.ijantimicag.2016.06.023.

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. https://doi.org/10.1128/CMR.00064-16.

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. https://doi.org/10.1016/j.tibs.2019.06.002.

17. Falagas ME, Rafailidis PI, Matthaiou DK. 2010. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Updat 13: 132–138. https://doi.org/10.1016/j.drup.2010.05.002.

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. https://doi.org/10.1128/AAC.01480-13.

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. https://doi.org/10.15252/emmm.201607336.

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. https://doi.org/10.1128/AAC.05052-14.

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 U S A 101:17162–17167. https://doi.org/10.1073/pnas.0406038101.

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. https://doi.org/10.1371/journal.pgen.1002184.

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. https://doi.org/10.1111/j.1365-2958.2010.07115.x.

24. Sato T, Shiraishi T, Hiyama Y, Honda H, Shinagawa M, Usui M, Kuronuma K, Masumori N, Takahashi S, Tamura Y, Yokota SJ. 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. https://doi.org/10.1128/AAC.00864-18.

25. Froelich JM, Tran K, Wall D. 2006. A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. J Bacteriol 188:1180–1183. https://doi.org/10.1128/JB.188.3.1180-1183.2006.

26. Cannatelli A, Giani T, Aiezza N, Di Pilato V, 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. https://doi.org/10.1038/s41598-017-05167-6.

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. https://doi.org/10.3389/fmicb.2017.02278.

28. Bourrel AS, Poirel L, Royer G, Darty M, Vuillemin X, Kieffer N, Clermont O, Denamur E, Nordmann P, Decousser J-W, IAME Resistance Group. 2019. Colistin resistance in Parisian inpatient faecal *Escherichia coli* as the result of two distinct evolutionary pathways. J Antimicrob Chemother 74:1521–1530. https://doi.org/10.1093/jac/dkz090.

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. https://doi.org/10.1038/nature07830.

30. Maeshima N, Fernandez RC. 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol 3:3. https://doi.org/10.3389/fcimb.2013.00003.

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. https://doi.org/10.1128/mBio.02317-17.

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. https://doi.org/10.3389/fmicb.2017.02262.

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 A-MW, 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. JAMA 312:1429–1437. https://doi.org/10.1001/jama.2014.7247.

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. https://doi.org/10.1128/mBio.00347-16.

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. https://doi.org/10.1016/j.jgar.2019.07.002.

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. https://doi.org/10.1038/s41467-018-03205-z.

37. Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. Emerg Infect Dis 22:1038–1043. https://doi.org/10.3201/eid2206.151840.

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. https://doi.org/10.1016/j.ijantimicag.2014.07.020.

39. Trent MS, Ribeiro AA, Doerrler WT, Lin S, Cotter RJ, Raetz C. 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. https://doi.org/10.1074/jbc.M106962200.

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. https://doi.org/10.1093/molbev/mss236.

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. https://doi.org/10.1099/ijsem.0.001572.

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. https://doi.org/10.1128/AAC.01016-08.

43. Schultz JE, Kanchan K, Ziegler M. 2015. Intraprotein signal transduction by HAMP domains: a balancing act. Int J Med Microbiol 305:243–251. https://doi.org/10.1016/j.ijmm.2014.12.007.

44. Lee J-Y, Choi M-J, Choi HJ, Ko KS. 2016. Preservation of acquired colistin resistance in Gram-negative bacteria. Antimicrob Agents Chemother 60:609–612. https://doi.org/10.1128/AAC.01574-15.

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. https://doi.org/10.1016/j.ijantimicag.2015.11.009.

46. van der Putten BCL, Matamoros S, COMBAT Consortium, Schultsz C. 2019. Genomic evidence for revising the *Escherichia* genus and description of *Escherichia ruysiae* sp. nov. bioRxiv https://www.biorxiv.org/content/10.1101/781724v2.

47. Son SJ, Huang R, Squire CJ, Leung I. 2019. MCR-1: a promising target for structure-based design of inhibitors to tackle polymyxin resistance. Drug Discov Today 24:206–216. https://doi.org/10.1016/j.drudis.2018.07.004.

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. https://doi.org/10.1038/msb4100050.

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. https://doi.org/10.1128/jb.185.16.4956-4972.2003.

50. McKenzie GJ, Craig NL. 2006. Fast, easy and efficient: site-specific insertion of transgenes into enterobacterial chromosomes using Tn7 without need for selection of the insertion event. BMC Microbiol 6:39. https://doi.org/10.1186/1471-2180-6-39.

51. Andrews JM. 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48:5–16. https://doi.org/10.1093/jac/48.suppl_1.5.

52. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, 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. https://doi.org/10.1089/cmb.2012.0021.

53. Loman NJ, Quinlan AR. 2014. Poretools: a toolkit for analyzing nanopore sequence data. Bioinformatics 30:3399–3401. https://doi.org/10.1093/bioinformatics/btu555.

54. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

55. Letunic I, Bork P. 2018. 20 years of the SMART protein domain annotation resource. Nucleic Acids Res 46:D493–D496. https://doi.org/10.1093/nar/gkx922.

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. https://doi.org/10.1093/jac/dks261.

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. https://doi.org/10.1186/s13059-014-0524-x.

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. https://doi.org/10.1093/molbev/mst197.

59. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic Local Alignment Search Tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

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. https://doi.org/10.1073/pnas.1508820112.

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. https://doi.org/10.1128/IAI.05226-11.

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. https://doi.org/10.1194/jlr.D500014-JLR200.

63. Sharma RC, Schimke RT. 1996. Preparation of electro-competent *E. coli* using salt-free growth medium. Biotechniques 20:42–44. https://doi.org/10.2144/96201bm08.

64. Silva D, Santos G, Barroca M, Collins T. 2017. Inverse PCR for point mutation introduction, p 87–100. Methods in molecular biology. Humana Press Inc., Totowa, NJ.

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. https://doi.org/10.1093/gbe/evt038.

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. https://doi.org/10.1099/ijs.0.02475-0.

67. Joint CLSI-EUCAST Polymyxin Breakpoints Working Group. 2016. Recommendations for MIC determination of colistin (polymyxin E). EUCAST, Växjö, Sweden. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf