Mechanisms of colistin resistance in *Escherichia* strains isolated from bloodstream infections

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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 colistin resistance mechanisms in ten colistin-resistant *Escherichia* strains out of 1140 bloodstream isolates, originating from patients hospitalised 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-resistant strains were acquired independently in each case. All colistin-resistant strains had lipid A that was modified with phosphoethanolamine. One strain carried the mobile colistin resistance gene *mcr-1.1*. Through construction of chromosomal transgene integration mutants, we experimentally determined that mutations in *basRS*, encoding a two-component signal transduction system, led to colistin resistance in four strains. While colistin resistance in *E. coli* can be acquired through *mcr-1.1*, sequence variation in *basRS* is another, potentially more prevalent but underexplored, cause of colistin resistance.
Introduction

*Escherichia coli* is a Gram-negative opportunistic pathogen that is a common cause of bloodstream, urinary tract, and enteric infections. 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. 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.

Colistin is a cationic, amphipathic molecule consisting of a non-ribosomal synthesized decapeptide and a lipid tail. Colistin specifically targets Gram-negative bacteria by binding to the anionic phosphate groups of the lipid A moiety of lipopolysaccharides (LPS) through electrostatic interactions. Colistin destabilizes the outer membrane, but the subsequent disruption of the inner membrane ultimately leads to cell death. Acquired colistin resistance has been reported in various Gram-negative bacteria that were isolated from clinical, veterinary, and environmental sources. 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. 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.

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. 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 confer colistin resistance in Salmonella and
Klebsiella confer resistance in E. coli\textsuperscript{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\textsuperscript{14,23}. This may explain why colistin resistance in clinical E. coli strains has only
been linked to mutations in basRS\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{11,14,31}.

Relatively little is known about colistin resistance mechanisms in E. coli, other than the
acquisition of mcr-genes\textsuperscript{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.
Material 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 microbiologic diagnostics 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 ∆ΔbasRS strain BW27848 from the Keio collection were obtained from the Coli Genetic Stock Center\(^{33,34}\). 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\(^{35}\). When appropriate, kanamycin (50 mg/L; Sigma-Aldrich, Zwijndrecht, The Netherlands), and ampicillin (100 mg/L; 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

Minimal inhibitory concentrations (MICs) to colistin were determined as previously described\textsuperscript{36} 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\textsuperscript{TM} 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 \textit{E. coli} was obtained from EUCAST (http://www.eucast.org/clinical_breakpoints/). \textit{E. coli} MG1655 served as a colistin-susceptible control.

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 dsDNA Broad Range Assay kit and the Qubit 2.0 fluorometer (Life Technologies, Bleiswijk, The Netherlands).

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 MiSeq system with a 500-cycle (2 × 250 bp) MiSeq reagent kit v2. 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 chromosomal DNA for 2 x 120 seconds at 1500 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 sequence data was assessed using FastQC v0.11.5 (https://github.com/s-andrews/FastQC). 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 using Illumina 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, minimum contig/scaffold average Nt coverage = 10-fold. MinION reads in FastQ format were extracted from Metrichor base-called FAST5-files using Poretools. Hybrid assemblies were generated of short- and long-read data using SPAdes by specifying Nanopore data with the --nanopore flag. Gene prediction and annotation was performed using Prokka. MLST typing was performed using the mlst package v2.10 (https://github.com/tseemann/mlst). Assembled contigs were assessed for antibiotic resistance genes using ResFinder 3.2.

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 1) using ParSNP v1.2. MEGA6 was used to midpoint root and visualize the phylogenetic tree. 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. Mutations that were identified in the genome sequences were confirmed through PCR (oligonucleotide primer sequences are provided in Supplemental Table 2) 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\textsuperscript{19,44,45}. Briefly, \textit{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\textsuperscript{46}. Mass spectrometry analyses were performed on a Bruker autoflex\textsuperscript{TM} 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 \textit{E. coli} strain MG1655 grown in LB medium at 37°C.

Prediction of functional impact of sequence variation in \textit{basRS}

We aimed to predict the functional impact of sequence variation in \textit{basR} and \textit{basS} using ConSurf\textsuperscript{47}, Scorecons\textsuperscript{48}, and PROVEAN\textsuperscript{49}. Multiple sequence alignments (MSAs) for BasR and BasS amino acid sequences were generated using ConSurf with standard settings. Using the BasR and BasS MSAs, ConSurf was used to determine the rate of conservation of each amino acid residue using the Bayesian calculation method, and JTT as evolutionary substitution model\textsuperscript{47,50}. Scorecons was used to quantify residue conservation, with consideration of the characteristics of each residue, using standard settings\textsuperscript{48}. These methods were used to score conservation of the specific residues, with highly conserved residues being more likely to importantly contribute to protein function. In addition to ConSurf and Scorecons, PROVEAN\textsuperscript{49} was used to predict the impact of the different BasR and BasS alleles on protein function compared to the publicly available \textit{Escherichia} reference genome sequences.
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\(^\text{35}\). 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 (Supplemental Table 2; 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\(^\text{35}\).

Electrocompetent BW25113 and BW27848 E. coli cells were prepared as described previously\(^\text{51}\) and transformed using the following settings: voltage 1800V, capacitance 25 µF, resistance 200Ω, 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 attTn7 site by PCR (primers listed in Supplemental Table 2) 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\(^\text{52}\). 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).
Statistical analysis

Statistical significance was determined using the non-parametric Kruskal-Wallis one-way, two-tailed ANOVA test. Correction for multiple comparison testing was performed using Dunn’s correction. Family-wise significance was defined as a p-value < 0.05.
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 automated antibiotic susceptibility profiles were available, and for which species identification had 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 MIC > 2 µg/ml colistin, was confirmed through broth microdilution (Fig. 1). Strain A783 was a false positive for colistin resistance during automated susceptibility testing in routine diagnostic procedures, and was excluded from further analysis, leaving ten isolates for further investigation (Table 1). 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, for varying indications (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. 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-resistant bloodstream *E. coli* isolates are not clonal

To assess the phylogenetic relationships between the colistin-resistant strains, a phylogenetic tree was generated based on the assembled contigs of the colistin-resistant strains and 210 publicly available complete genome sequences (Supplemental Table 1). Based on a core genome alignment of 874 kbp, we did not observe direct transmission of colistin-resistant strains between patients (Fig. 2a). Three colistin-resistant strains (strains I1121, H2129, and G821) belonged to the globally
disseminated ST131 clone (Fig. 2a), and all three were dispersed throughout the multidrug-resistant clade C of ST131\textsuperscript{3,54}. This indicates that these ST131 strains have independently acquired colistin resistance (Fig. 2b). Strain A2361 clustered among \textit{E. albertii}, although it had been typed as \textit{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 \textit{mcr} gene \textit{mcr-1.1} (0.086\% of all bloodstream isolates; Supplemental Fig. 1). After long-read sequencing and hybrid assembly, the \textit{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 \textit{mcr-1.1} carrying IncX4-type plasmid from E3090 shares 99\% identity to the previously reported \textit{mcr-1.1} carrying IncX4-type plasmid pMCR-1_Msc (GenBank accession MK172815.1) harboured by \textit{E. coli} isolated from patients in Russia\textsuperscript{55}, confirming the global dissemination of this plasmid\textsuperscript{56}. In all strains studied here, a variety of acquired resistance genes was observed (Supplemental Fig. 1), 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 strain A2361 did not possess any acquired resistance genes.

\textit{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 \textit{E. coli} we extracted lipid A from the clinical strains and the colistin-susceptible control \textit{E. coli} strain MG1655, and subjected them to MALDI-TOF mass spectrometry. The lipid A produced by all \textit{E. coli} strains showed lipid A species with a mass-to-charge ratio (\textit{m/z}) of 1797, corresponding to the canonical unmodified \textit{E. coli} hexa-acylated lipid A (Fig. 3). Colistin-resistant strains showed additional lipid A species at \textit{m/z} 1921, consistent with the addition of phosphoethanolamine (\textit{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. alberti* 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$_{14}$. 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 correspond to the loss of the second phosphate group, compared to $m/z$ 1948, and $m/z$ 2187.

**Identification of candidate mutations involved in colistin resistance.**

As mutations in *basRS* have been suggested to cause to 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 (Supplemental Fig. 1), 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. Since BW27848 still possesses the gene encoding for the phosphoethanolamine transferase EptA, we constructed sequences that consisted of the fused sequences of the promotor region of the *eptA-basR-basS* operon and the *basRS* coding sequences in order to prevent *eptA* gene dose-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 significantly higher colistin MIC values than
the BW27848::Tn7-empty strain (adjusted p-values of 0.0195, 0.0094, 0.0008, and 0.008 respectively), with observed MIC values higher than the 2 µg/ml cut-off for resistance as set by EUCAST (Fig. 4). 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 cause the found 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 Fig. 2. 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 duplication of the HAMP-domain in BasS in G821, and a A159P substitution in BasS in Z821 (Fig. 5). As expected, in the mcr-1.1 positive strain E3090 no mutations in basRS were identified.

To assess the possible role of observed variations in basRS in colistin resistance, we used ConSurf47 and Scorecons48 to score conservation of the specific residues. In addition, we used PROVEAN49 to predict the impact of the mutations on protein function. As input sequences, we used the BasR and BasS amino acid sequences of the genomes that most closely matched to the colistin-resistant strains (Fig. 2), as this allowed the scoring of the identified substituted residues. The rate of conservation of an amino acid indicates its importance in the protein, with higher conserved residues being more likely to be important for correct protein functioning. ConSurf results indicated that one of the three substituted residues, A159 in BasS, was highly conserved (Fig. 5). In contrast, Scorecons shows that the specific characteristics of all three affected residues were conserved. The
PROVEAN results suggested that the G53S substitution in BasR, and the duplication of the HAMP-domain and A159P substitution in BasS could impact protein function. ConSurf or Scorecons could not be used for the analysis of the observed duplication in strain G821. Thus, for all observed mutations, an impact of the substitution of the residues on protein functioning was supported by at least one method.

Using these *in silico* prediction methods, 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 (Fig. 5) were causal to the development of colistin resistance, the identified mutations were reversed through site-directed inverse PCR mutagenesis to match the publicly available genome sequence. The MIC values of these mutants returned to levels similar to that of the colistin-susceptible BW27848::Tn7-empty strain (Fig. 6). These experiments support the *in silico* predictions on the functional impact of the *basRS* sequence variations observed in the colistin-resistant *E. coli* strains.
Discussion

In the present study, we identified the mechanisms through which *E. coli* bloodstream isolates can develop colistin resistance. 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 ∆*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*\(^{36}\). The glycine in position 53 of BasR has previously been reported to be altered in colistin-resistant Enterobacteriaceae\(^{57,58}\) including in *E. coli*\(^{59}\). The G53S change specifically, as in isolate H2192, has been experimentally proven to contribute to colistin resistance in *Klebsiella* (previously *Enterobacter*) *aerogenes*\(^{60,61}\) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium\(^{62}\) 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\(^{63}\). 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\(^6^4\), and in clinical colistin-resistant E. coli isolates\(^6^5\), 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\(^6^6\).

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\(^1^4\). 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. 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\(^3^1\) could be a target for future drug development and opens the possibility of combination therapy with colistin and an inhibitor of phosphoethanolamine transferase\(^6^7\). 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.
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Author contributions

A.B.J. conceived and designed experiments, performed experiments, analysed data, and wrote the manuscript. T.B.L. 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.
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Table 1: Overview of colistin-resistant bloodstream isolates used in this study.

| Strain | MLST | Date of isolation | Received colistin <3 months before isolation of strain | Reason for colistin treatment | Route of administration |
|--------|------|-------------------|--------------------------------------------------------|-------------------------------|--------------------------|
| I1121  | 131  | 22-4-2015         | Yes                                                    | P. aeruginosa colonisation of lungs (cystic fibrosis patient) SOD treatment | Inhalation (treatment of P. aeruginosa colonisation); oral (SOD treatment) |
| H2129  | 131  | 22-7-2014         | No                                                     | N.A.                          | N.A.                     |
| G821   | 131  | 19-3-2013         | No                                                     | N.A.                          | N.A.                     |
| F2745  | 73   | 2-11-2012         | No                                                     | N.A.                          | N.A.                     |
| E3090  | 10   | 12-11-2011        | No                                                     | N.A.                          | N.A.                     |
| E2372  | 59   | 25-8-2011         | No                                                     | N.A.                          | N.A.                     |
| E650   | 162  | 11-3-2011         | No                                                     | N.A.                          | N.A.                     |
| D2373  | 6901 | 20-10-2010        | Yes                                                    | Colistin due to relapsing infections related to common immunodeficiency; SDD treatment in hematology ward due to allogeneic stem cell transplantation | Oral                     |
| A2361  | 5268 | 3-11-2007         | No                                                     | N.A.                          | N.A.                     |
| Z821   | 167  | 2-4-2006          | Yes                                                    | SDD treatment in hematology ward due to allogeneic stem cell transplantation | Oral                     |

Overview of the used isolates in this study, including for each isolate the MLST type, date of isolation, information on the use of colistin three months before the isolation of the colistin-resistant *Escherichia* isolate, and if applicable, indication and route of administration. N.A., not applicable.
**Figures and legends**

**Figure 1: Colistin MICs of nosocomial bloodstream isolates.**

Colistin MIC determination by microbroth dilution of eleven nosocomial *Escherichia coli* bloodstream isolates included in this study and *E. coli* MG1655. The epidemiological cut-off value (ECOFF) for colistin resistance (2 µg/ml) by EUCAST is depicted with a dashed line. The values presented represent the mean (± standard deviation) of three independent replicate experiments performed in triplicate.
Figure 2: Colistin-resistant strains are not clonally related.

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. The different branches of E. coli and E. albertii are indicated with blue and green arcs, respectively.
ST131 lineage of *E. coli* is indicated by a yellow arc. 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 arcs respectively.
Figure 3: 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.
Figure 4: Chromosomal transgene insertion mutants of basRS alleles from resistant strains highlight the importance of basRS for the development of colistin resistance in E. coli.

E. coli strain BW27848 is the ΔbasRS mutant of BW25113. The basRS alleles of colistin-resistant strains from this study were inserted into the attTn7 site of BW27848. ECOFF for colistin resistance is defined as an MIC value higher than 2 µg/ml by EUCAST, and is depicted with a dashed line. The values presented represent mean (± standard deviation) of three independent replicate experiments performed in triplicate. Statistical testing was performed by comparing the strains carrying a basRS allele, BW27848::Tn7-empty strain, that harbours an insertion of the unmodified Tn7 transposon at the attTn7 site in the BW27848 background. Statistical testing was performed using the non-parametric Kruskall-Wallis, one-way, two-tailed ANOVA test, with Dunn’s correction for multiple comparisons. Significance was defined as a P-value < 0.05 (*), < 0.01 (**), or <0.001 (***)}. Family-wise significance was defined as a p-value < 0.05.
Figure 5: 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\(^\text{68}\). 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. The sites at which colistin-resistant strains had non-synonymous mutations compared to their reference strains were subjected to ConSurf and Scorecons analyses to determine rate of conservation of the residue found in the closest matching publicly available genome sequence, and PROVEAN analysis for the impact of the mutation on protein function. The degree of conservation is rated from completely conserved to not conserved, whilst PROVEAN analysis classifies the effect of mutations as neutral of deleterious. Results of the analyses are coloured according to the given legends. N.A., not applicable.
Figure 6: Chromosomal transgene insertion mutants of mutated basRS alleles show impact of the identified basRS mutations on development of colistin resistance in E. coli.

E. coli strain BW27848 is the ∆basRS mutant of BW25113. The basRS alleles of colistin-resistant strains from this study were inserted into the attTn7 site of BW27848. Strain names with a “m” indicates the construct in which the previously identified mutation (Fig. 5) has been reversed through inverse PCR site-directed mutagenesis. ECOFF for colistin resistance is defined as an MIC value higher than 2 µg/ml by EUCAST, and is depicted with a dashed line. The values presented represent mean (± standard deviation) of three independent replicate experiments performed in triplicate. Statistical testing was performed by comparing the strain carrying a basRS allele in which the found mutation was reversed, with the strain with the basRS allele from the clinical strain and the BW27848::Tn7-empty strain, that harbours an insertion of the unmodified Tn7 transposon at the attTn7 site in the BW27848 background. Testing was performed with the non-parametric Kruskall-Wallis test, with Dunn’s correction for multiple comparisons. Significance was defined as a
p-value < 0.05 (*), < 0.01 (**), or <0.001 (***)). Family-wise significance was defined as a p-value < 0.05.