Resolving colistin resistance and heteroresistance in Enterobacter species

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Article

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Abstract:

Enterobacter cloacae complex (ECC) represent globally important nosocomial pathogens. A three-year study of ECC in Germany identified *Enterobacter xiangfangensis* as the most common species (65.5%) detected, a result replicated by examining a global pool of 3246 isolates. Antibiotic resistance profiling revealed widespread resistance and heteroresistance to the antibiotic colistin and detected the mobile colistin resistance (*mcr*)-9 gene in 19.2% of all isolates. We show that resistance and heteroresistance properties depend on the chromosomal *arnBCADTEF* gene cassette whose products catalyze transfer of L-Ara4N to lipid A. Using comparative genomics, mutational analysis, and quantitative lipid A profiling we demonstrate that intrinsic lipid A modification levels are genospecies-dependent and governed by allelic variations in *phoPQ* and *mgrB*, that encode a two-component sensor-activator system and specific inhibitor peptide. By generating *phoPQ* chimeras and combining them with *mgrB* alleles, we show that interactions at the pH-sensing interface of the sensory histidine kinase *phoQ* dictate *arnBCADTEF* expression levels. To minimize therapeutic failures, we developed an assay that accurately detects colistin resistance levels for any ECC isolate.

Introduction:

Members of the order *Enterobacterales* are a frequent source of morbidity and mortality associated with bloodstream, respiratory tract, and urinary tract infection in healthcare institutions worldwide[1,2]. Among the *Enterobacterales*, *Enterobacter* are opportunistic nosocomial pathogens listed among the top five species causing bloodstream infections (BSI)[3–5], and are in WHO's priority list for which the new antimicrobials are urgently needed[6]. *Enterobacter* are also present in environmental habitats such as soil and water and exhibit considerable phenotypic and genomic diversity[7]. As a result of taxonomical complexity, pathogenic species are commonly grouped

78 within the *Enterobacter cloacae* complex (ECC)[8]. Infections with *Enterobacter* species are
79 difficult to treat as they often exhibit resistance to penicillin, quinolones, and third-generation
80 cephalosporins[9]. While carbapenems are among the most attractive therapeutic options,
81 *Enterobacter* species have emerged as third most common hosts for carbapenemases worldwide
82 forcing the use of colistin as a 'last-resort' antibiotic for the treatment[10–12].

83 Colistin and other cationic peptides are membrane-active agents that target the
84 lipopolysaccharide (LPS) component of the bacterial outer membrane (OM)[13]. Colistin is often
85 considered a last-line therapeutic option for treating *Enterobacter* infections, but the prevalence of
86 resistance among *Enterobacter* species is uncertain. MIC determinations show high variability
87 depending on the test methods used[14]. The WHO Global Antimicrobial Resistance Surveillance
88 System (GLASS) reports *Enterobacter* species as being naturally susceptible to colistin [15,16]. Other
89 studies indicate that resistance towards colistin is either rare or can comprise up to 17% of all
90 *Enterobacter* species[17,18]. A recent systematic study of *Enterobacterales* found that ~20%
91 *Enterobacter* isolates were colistin resistant, as compared to <2% isolates for *E. coli* and
92 *Klebsiella*[17]. *Enterobacter* isolates frequently exhibit heteroresistance to colistin making accurate
93 resistance testing difficult[19]. These complications often lead to high risk of treatment failures even
94 with isolates that have previously been classified as susceptible[19,20].

95 In this study we performed a genome-based taxonomic study on clinical isolates of *Enterobacter*
96 obtained over a three-year period from six university hospitals at different locations in Germany.
97 Our data suggest temporal and geographic predominance of the species *E. xiangfangensis*, a
98 finding that was replicated by examining whole genome sequences of over 3,246 isolates from
99 clinical sources worldwide. In contrast to previous reports, we find that colistin resistance and
100 heteroresistance in the genus *Enterobacter* is widespread, involving over 80% of all known

species. As the problem of heteroresistance and its impact on unexplained treatment failures of clinical infections are of great concern, we applied phylogenomics, antimicrobial susceptibility testing, lipid A profiling together with the isogenic deletion mutation and complementation analysis, to reveal mechanisms that characterize colistin heteroresistance in *Enterobacter*.

Methods:

Ethics:

The study was approved by the ethics committee of the coordinating site (Cologne) and all sampling sites (Ethical committee approval, coordinating site Cologne: EA4/018/14).

Isolates and antibiotic resistance profiles

Bacterial strains used in this study are listed in Supplementary data 1. Cultures were grown in Luria-Bertani (LB) broth (per liter composition: 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7) (BD Difco™ Dehydrated Culture Media).

A total of 480 clinical *Enterobacter* isolates (bloodstream, n = 408 and colonization n = 72) were obtained between 2016 and 2018 from six university hospital centers (Berlin, Lübeck, Cologne, Gießen, Tübingen, and Freiburg) within a surveillance project targeting multidrug-resistant bacterial organisms (R-Net: https://www.dzif.de/en/node/915). Bloodstream infection isolates were obtained through routine diagnostics following clinical indication. Colonization isolates were obtained from newly admitted patients (<48 h hospitalization) on selective agar plates for resistant isolates. Of 480 isolates, 141 were randomly selected (~ one-third from each site) and supplemented with 24 historical isolates circulating in Germany (between year 2011-2015, random selection). A total of 165 isolates (bloodstream infections (n = 96) and rectal swabs (n = 69)) were sequenced and studied (Supplementary data 1). These isolates were identified as *Enterobacter* spp. using MALDI-TOF (VITEK MS) or the VITEK2® GN ID card (bioMérieux, France). Antibiotic resistance phenotypes were determined using the VITEK2® system.

Colistin resistance was determined by calculating minimum inhibitory concentrations (MIC) using the broth microdilution (BMD) method following CLSI-EUCAST Polymyxin

Breakpoints Working Group recommendations[21]. In brief, 10 µL from a 30% glycerol stock (in LB broth) was revived in 2 mL LB broth (in pre-sterilized 14 ml PP tube, Greiner Bio-One, Cat. No. 187261) by incubation at 37 ºC for 18 h at 180 rpm. The culture was diluted 1:100 in fresh 5 mL LB broth (in 14 ml PP tube, Cat. No. 187261) and incubated at 37 ºC at 180 rpm till the $O.D._{600}$ reached to 0.8 to 1.0, following which cultures were used for BMD assay. For BMD, 10 µL inoculum were added to 190 µL of LB broth containing a range of 512 to 0.5, and 0 mg/L colistin in 96-well plate using a pipetting robot (Assist Plus, Integra). Plated were incubated without shaking at 37 ºC for 16-20 h, and readings were taken by Tecan 96-well plate reader (Infinite M200 Pro, Tecan Group Ltd. Maennedorf, Switzerland) at 600 nm. To determine the MICs at the various growth pH used, the pH of the LB broth was adjusted using 1 M HCl or NaOH. MIC experiments were repeated three times. *E. coli* DH10β was used as control in each plate.

Whole-genome sequencing, genome-based taxonomy, and comparative genomic analysis:

For whole-genome sequencing, bacterial DNA was isolated from overnight cultures using the PureLink Genomic DNA isolation kit (Thermo Fisher Scientific, Germany) and libraries were prepared using the Nextera XT kit (Illumina, The Netherlands), individually tagged libraries and sequenced (2x300 bp/2x150 bp) on the MiSeq/NextSeq platform (Illumina)[22]. For ten isolates harboring the *mcr-9* gene, long-read sequences were obtained. The library was prepared using the native barcoding kit (EXP-NBD103, Oxford Nanopore Technologies) and 1D chemistry (SQK-LSK108, Oxford Nanopore Technologies). Sequencing was performed on a MinION sequencer (Oxford Nanopore Technologies) using a SpotON Mk I R9 Version flow Cell (FLO-MIN106, Oxford Nanopore Technologies). Hybrid *de novo* genome assembly was performed using Unicycler v0.4.8[23]. Genome sequences are deposited in the public database under the BioProject accession number PRJNA622426.

Raw-read quality control, assembly and annotation were carried out as described earlier (detailed in Supplementary data 1)[22]. *Enterobacter* species were identified based on the 'overall genome related index' (OGRI)[24] and included 23 *Enterobacter* species valid as of June 2022[25,26]. The OGRI was determined by calculating the average nucleotide identity (ANI) and *in silico* DNA-DNA hybridization score. The ANI was calculated by BLAST using JSpecies v1.2.1 tool[27]. As the pairwise comparison by JSpecies may differ slightly and is problematic for borderline species[28], we also obtained ANI using the two-way approach from the Enveomics package (http://enve-omics.ce.gatech.edu/enveomics/) (with identical settings of JSpecies v1.2.1 alignment program: blast, window size 1020 bp, step size 150 bp, minimum alignment length 714 bp (70%), minimum alignment identity 306 bp (30%)). The *in silico* DNA-DNA hybridization score was calculated by genome-to-genome distance calculator (GGDC) using formula-2, respectively[29].

Phylogenomic grouping was carried out by multiple approaches such as concatenated core genes by Roary v3.12.0 combined with RaxML v8.2.11[30,31], the core genome by Harvest suite v1.2[32], and for the whole genome, kSNP3.0[33]. Bayesian Analysis of Population Structure (BAPS) clusters were determined using RhierBAPS[34]. The lineages were define based on the concordance of bootstrap supported phylogenomic grouping and BAPS clusters (assignment probability >0.99). Lineage-specific genes were identified by Scoary v1.6.16[35]. The *hsp60* typing was carried out based on the grouping of partial *hsp60* gene sequence to that of Hoffmann's hsp60 gene sequence[36]. Phylogenomic analysis for worldwide isolates was performed using kSNP3.0[33] as well as through using genomic distance as obtained through MASH v2.1.1[37]. Phylogenomic tree and associated metadata was visualized in iTOL v4[38]. Genetic structures were visualized in Easyfig v2.1[39]. Plasmid incompatibility groups and the respective plasmid multilocus sequence typing was performed using PlasmidFinder and pMLST software tools, respectively[40].

Single nucleotide variants (SNVs) were identified by mapping filtered reads against the completely closed ESBL3012 reference genome using snippy v4.3.6 (https://github.com/tseemann/snippy). Pairwise genomic comparison and visualization was carried out in Ugene v1.32.0 and the 'gene presence/absence' output of Roary, and pan-genome-wide association studies were carried out with Scoary. Plasmid replicons, prophages, virulence, and antibiotic resistance genes were determined by screening for the presence of genes in the respective databases (see Supplementary methods).

Plasmid and prophage genes were identified by BLAST alignment (70% query coverage and 85% nucleotide identity cutoff) against plasmid[41] and prophage sequences databases (http://mvp.medgenius.info/phages), respectively. Virulence gene profiling was performed by aligning amino-acid sequences of all genes (using 70% query coverage and 80% nucleotide identity) against the 'Virulence Factor Database' (VFDB) using the Diamond v0.8.36.98 tool[42]. Antibiotic resistance gene profiling was carried out using NCBI's AMRfinder (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/) as well as by 'Resistance Gene Identifier' v5.05.5 against CARD database (v3.0.3)[43]. For mobile colistin resistance gene (*mcr*), data on clinical and environmental isolates was compiled from NCBI's Pathogen Detection pipeline (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/).

Comparative analysis of genome sequences of *Enterobacter* spp. obtained worldwide

For comparative genome-based analyses, whole-genome sequences of 3246 non-repetitive isolates (<1000 contigs and >3 Mb assembly size) listed under the genus *Enterobacter* were downloaded from NCBI using e-utilities (Supplementary data 2). These isolates were obtained from clinical, non-clinical, and unknown sources from more than 67 countries. These isolates were

selected after reconfirming them as *bona fide Enterobacter* species using OGRI tool (as detailed in Supplementary methods).

Population analysis profiling (PAP) test

PAP tests were carried out for all the 165 isolates. Isolates were grown as described for MICs. Serial dilutions of culture with $O.D._{600}$ 0.8-1.0 were prepared and 100 µL culture (from 10^{-4}, 10^{-6}, and 10^{-8} dilutions) was plated on LB agar plates containing either 0, 8, or 32 mg/L colistin [44]. The plates were incubated at 37 ºC for 24 hours. CFUs were counted using an automatic colony counter (Scan 500, Interscience) and heteroresistance frequency was calculated by estimating 'CFUs recovered on plate containing colistin' x 100 / 'CFUs on plate without colistin'.

Mutant generation and complementation

The mutants for *arnBCADTEF*, *pmrAB*, *phoPQ*, and *mgrB* genes were generated by homologous recombination using the λ-Red recombination system[45]. The vector pSIM5-tet carrying the λ-Red genes (γ, β and exo) was introduced into the strain RBK-17-0394-1[46]. Next, a PCR product for kanamycin resistance gene flanked by FRT-sites (FLP recognition target) was generated from vector pKD4. The transformants carrying the λ-Red recombination system (pSIM5-tet) were grown in LB media containing tetracycline at 30 °C to $O.D._{600}$ of 0.4. Thermal induction of λ-Red recombination system was carried out by incubating transformant at 42 °C for 15 min. The λ-Red recombination system (pSIM5-tet) transformants grown to an $O.D._{600}$ of 0.6 were made electrocompetent by washing cells (3 times) with ice-cold 10% glycerol. After electroporation of the linear PCR product the mutants were selected on the agar plate containing 50 mg/L kanamycin at 30 °C. The vector pSIM5-tet was eliminated by growing cells at 42 °C. All the primers and vectors used are listed in the Supplementary data 3. For complementation, respective genes with their original promoter region (-60 to -100 bp) were PCR-amplified and

cloned in a low-copy broad host range vector pBBR1-MCS2. Recombinant plasmids were transformed in the respective mutants. Hybrid *phoP/phoQ* mutants were generated by 'Gibson Assembly Cloning Kit' (NEB, #E5510S) following manufacturer's instructions.

Lipid A extractions

Lipid A was isolated from freshly harvested bacterial cells using ammonium hydroxide-isobutyric acid extraction[47]. The cells were grown as described for MIC testing. At an $O.D._{600}$ of between 0.8 and 1.0, 5 mL culture were pelleted and resuspended in 400 µL of 70% isobutyric acid and 1 M ammonium hydroxide (5:3, [v/v]) followed by an incubation for 2 h at 100 °C in a screw-cap test tube. The mixture was cooled on ice and centrifuged at 2,000 x g for 15 min at 4 ºC. The resulting supernatant was diluted with water (1:1, [v/v]) and lyophilized (Thermovac TM201; Leybold-Heraeus, Cologne, Germany). The sample was washed with 1 mL of methanol and centrifuged at 2,000 x g for 15 min at 4 ºC. The insoluble lipid A fraction was solubilized and extracted using 100 µL of a mixture of chloroform:methanol:water (3:1.5:0.25, [v/v/v]) and dried prior to further analysis.

Mass spectrometry

All mass spectrometric analyses were performed on a Q Exactive Plus (ThermoFisher Scientific, Bremen, Germany) using a Triversa Nanomate (Advion, Ithaca, NY, USA) as nano-ESI source. Lipid A extracts were initially dissolved in 20 µL chloroform:methanol:water (60:30:5, [v/v/v]) or chloroform:methanol (8:2, [v/v]). 5 µL of such a solution were mixed with 95 µL of water/propan-2-ol/7 M triethylamine/acetic acid (50:50:0.06:0.02, [v/v/v/v]). In those cases where resulting solutions were too viscous for spraying, 1:10 dilutions in water/propan-2-ol/7 M triethylamine/acetic acid (50:50:0.06:0.02, [v/v/v/v]) were used for the measurement. Mass spectra

were recorded for 0.50 min in the negative mode in an *m/z*-range of 400-2500 applying a spray voltage set of -1.1 kV. All depicted mass spectra were charge deconvoluted (Xtract module of Xcalibur 3.1 software (ThermoFisher Scientific, Bremen, Germany)) and given mass values refer to the monoisotopic masses of the neutral molecules, if not indicated otherwise. For the calculation of the ratios of non-modified and L-Ara4N-modified lipid A species mass spectrometric data of 143 acquisitions including 6 blanks were imported into one database using LipidXplorer 1.2.8[48]. Monoisotopic peaks of 57 lipid A species were calculated and assigned. Intensities of all observed tetra- to hepta-acylated lipid A species were considered and utilized to determine substitution ratios. Details on the lipid A molecular species, detected ions, and calculation of lipid A species relative abundance can be found in Supplementary data 4.

Fluorescence microscopy

Bacteria were grown in conditions as used for the MIC test. From freshly grown cultures ($O.D._{600}$ 0.8-1.0), 2 mL culture was pelleted (8,000 x g for 10 min) and resuspended in 0.85% NaCl. The $O.D._{600}$ was adjusted to 0.1 (~ 10^8 CFUs/mL) and two sets (1 mL each) were created as treatment and control. For treatment, colistin sulphate was added to the final concentration of 64 mg/L and incubated at room temperature for 15 min. Cells were pelleted, washed twice, and resuspended in 0.85% NaCl solution. To determine the cells with the intact and compromised cell membranes, we used LIVE/DEAD™ BacLight™ Bacterial Viability Kit (ThermoFisher, # L7012). Equal volumes of component A (SYTO 9, 3.34 mM) and component B (Propidium iodide, 20 mM) was mixed, and 3 µL of dye mixture was added to 1 mL of cell suspension and incubated for 15 min in dark. Cell membrane integrity was also evaluated using FM4-64 (AAT Bioquest, #21487) (that stain membranes red) combined with DAPI (that following entry stains DNA blue). FM4-64 and DAPI was added to the final concentration of 2 and 1 mg/L, respectively, and

incubated for 15 min. Labelled bacterial suspensions (5 µL) was visualized under the fluorescence microscope (Keyence Biozero BZ-8000K). For LIVE/DEAD™ staining, cells with green fluorescence were considered as cells with intact membrane, while those with red fluorescence were considered as membrane- compromised cells.

RNA extraction, cDNA synthesis, and Quantitative PCR

Bacteria were grown as described for the MIC test. Once the $O.D._{600}$ of freshly grown cultures was reached 0.8-1.0, 1 mL of culture was mixed with RNAprotect reagent and stored at -80 ^{o}C. For RNA isolation, the mixture was defrosted on ice and pelleted by centrifugation (8,000 x g, 5 min at 4 ^{o}C). The pellet was processed by RNeasy Mini Kit (Qiagen, #74124) following manufacturer's protocol. The cDNA synthesis was performed using SuperScript II (Invitrogen) using 100 ng of total RNA with random hexamer and nonamer primers. Quantitative PCR amplification was performed by using 1 µL aliquot of a 1st strand cDNA reaction with brilliant SYBR Green qPCR master mix (Qiagen) on the OnePlus real-time cycler (Applied Biosystem) in a final volume of 25 µL. Specificity of all the amplicons was confirmed by gel analysis and melting curves. Experiments were performed in triplicate for three biological replicates. 16S rRNA was used as an endogenous control. Relative quantification was performed using the $2(-\Delta\Delta Ct)$ method[49]. The primers used are listed in Supplementary data 3.

Computational modeling of PhoQ and MgrB

To compute interactions between PhoQ sensor protein of *E. xiangfangensis* RBK-17-0394 (WT), *E. roggenkampii* RPB-17-0516-2 (516), and *E. bugandensis* RBG-17-0246-1 (246) with MgrB, we used reiterative homology modeling and molecular docking techniques. Homology-based PhoQ models were built using computationally build PhoQ[50], as a template structure

285 (https://salilab.org/phoq) through Swiss-model server[51]. To model the PhoQ periplasmic, trans-
286 membrane and HAMP region 3BQ8.pdb was used for periplasmic domain, 1H2S.pdb was
287 employed for trans-membrane domain, and 2ASW.pdb for HAMP region[50]. The atomic structure
288 of MgrB were modeled using the AlphaFold server[52]. The stereo-chemical qualities of modeled
289 structures of PhoQ and MgrB were evaluated using Ramachandran Plot through the
290 PROCHECK[53]. To explore the interaction of C-terminal periplasmic region of MgrB with
291 periplasmic domain (i.e. acidic region) of PhoQ, molecular docking were performed using
292 HADDOCK[54]. The analysis and visualization of molecular interactions were performed using the
293 PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LL.

Results:

Enterobacter xiangfangensis lineage-1 as the most common *Enterobacter* in clinical samples

Based on MALDI-TOF and VITEK data, the majority (86.0%) of the isolates were identified as *E. cloacae,* 8.4% as unknown *Enterobacter* species, 3.0% as *E. asburiae,* and 1.2% as *E. cloacae* complex, a single isolate each as *E. cancerogenus* and *E. ludwigii.* To study taxonomic assignments in greater details, we sequenced and studied one-third of isolates from each center (n=165, 96 from BSI and 69 from body sites) (Supplementary data 1b).

We calculated the 'overall genome relatedness index' (OGRI) of isolates by comparing them to type strains of 23 valid *Enterobacter* species as of June 2022 (Supplementary data 5). OGRI-based genome analysis identified 12 *Enterobacter* species from 165 isolates. Of these, a total of 108 (65.5%) were identified as *E. xiangfangensis,* 23 (13.9%) as *E. ludwigii*, and 9 isolates each as either (5.4%) *E. kobei* or *E. roggenkampii* (also see Supplementary results for *E. xiangfangensis/hormaechei* taxonomical conflict within Supplementary Material). The species *E. bugandensis, E. cloacae, E. asburiae, E. chengduensis, E. mori, E. wuhouensis*, *E. cancerogenus*, and *E. vonholyi* were observed only sporadically (≤5 isolates each) (Figure 1a). In total, 67 of 96 (69.8%) isolates from BSI were *E. xiangfangensis,* followed by *E. ludwigii* (12.5%) (Supplementary Table 1).

Core genome-based phylogenomics analysis together with Bayesian Analysis of Population Structure (BAPS) distributed *E. xiangfangensis* isolates into four major lineages, that we assign as Lineages (L)-1 to -4 (Figure 1b). A total of 64 of 108 *E. xiangfangensis* observed (59.2%) were members of L-1 isolates, while L-2, L-3, and L-4 were represented by 26, 11, and 7 isolates, respectively.

We used the OGRI-based approach to clarify the taxonomy of the publicly available genomes based on high-quality assemblies of *Enterobacter* from 67 countries. A total of 2233 from 3246 (68.7%) isolates deposited as ECC or *Enterobacter* species were identified as *E. xiangfangensis* (Supplementary data 2). Country-wise analysis (comprising 35 countries for which more than five genome sequences were available) indicated that *E. xiangfangensis,* particularly isolates of L-1, was the predominant species detected in 29 countries and comprised between 61 to 100% of all *Enterobacter* isolates reported therein (Figure 1c, Supplementary Table 2).

High occurrence of *mcr-9* gene in *E. xiangfangensis*

Antibiotic resistance gene profiling of isolates from Germany revealed 9 (8.3%) *E. xiangfangensis,* 2 *E. ludwigii* and a single isolate of *E. kobei* and *E. chengduensis* each, which carried the mobile colistin resistance (*mcr)*-9 gene (Figure 1b). Presently, an average of 2.5% (0.1-5.1%) of clinical isolates obtained from various infections is reported to carry *mcr* alleles[55] (Supplementary Table 3). This contrasts with the relatively high (8.3%) number of clinical *E. xiangfangensis* with the *mcr-9* gene in this study. Analysis of worldwide isolates revealed a total of 24.8% of *E. xiangfangensis* isolates harbor the *mcr-9* gene, while the presence of other *mcr* alleles (*mcr-1, 3, 4,* and *10*) was rare (up to 1.4%) (Supplementary data 2, Supplementary Figure 1). The BMD assay showed that colistin resistance in studied *Enterobacter* isolates was not affected and regardless of whether the *mcr-9* gene was present.

Colistin resistance and heteroresistance is associated with the presence of *arnBCADTEF*

Comparative genomics of colistin-susceptible versus -resistant isolates showed that resistance patterns associated strongly with the presence of the *arnBCADTEF* gene cassette (*arn*) (Table 1). Isolates of *E. wuhouensis, E. xiangfangensis* sub-lineages L-2, L-3 and L-4, and ST133

isolates of *E. xiangfangensis* L-1 that lacked *arn* showed colistin MIC ≤ 4 mg/L. The remaining isolates of *E. xiangfangensis* L-1 and all other species with *arn* exhibited MIC >4 mg/L (Table 2, Supplementary data 1b). Notably, for the *arn* encoding isolates, the MICs for colistin varied between 4 to ≥512 mg/L in a species dependent manner. Isolates of *E. xiangfangensis* (L-1), *E. asburiae, E. ludwigii, E. bugandensis, E. cancerogenus, E. chengduensis, E. cloacae, E. kobei*, and *E. roggenkampii* had MICs varying between 2 to ≥512 mg/L. A single isolate of *E. vonholyi,* that encoded *arn,* was observed to be sensitive (due to truncated regulator component *phoQ*; detailed below).

In repeated testing for colistin susceptibility using the BMD method, for the same isolate, two- to four-fold changes in the MIC with the "skip wells" phenomenon was observed indicating heteroresistance towards colistin[56]. Using the PAP assay, we calculated heteroresistance frequency (HRF) i.e., percentage of cells grown on LB agar plate containing colistin as compared to the inoculum used for all the species encountered. Only those isolates that carry an intact *arn/phoPQ* system exhibited heteroresistance, while isolates lacking this system or carrying truncated versions, showed neither the "skip well" phenomenon nor exhibited heteroresistance in PAP assay (Table 1). This data indicated, as with colistin resistance, that an intact *arn/phoPQ* system is required also for colistin heteroresistance.

We examined for the presence of *arn* in *Enterobacter* in additional species that were not detected in this study. Screening of genomes of *Enterobacter* species retrieved from public databases revealed that, of the 23 *Enterobacter* species known today (July 2022), 19 carry the *arn* gene cassette (Supplementary data 3). The location of this cassette is always found between the same core genes on the chromosome (Supplementary Figure 2). In those species lacking *arn*, there is a clean excision of this gene cassette leaving the same flanking genes intact. In summary, all the

isolates of *E. xiangfangensis* L-2, L-3, and L-4, *E. hormaechei*, *E. wuhouensis*, and a single isolate (i.e. type strain) of *E. timonensis* lack *arn.* These isolates lacking *arn* form a unified clade (Figure 1b, Supplementary Figure 1). As *Enterobacter* species carried *arn* at an identical chromosomal location and were members of a single phylogenomic clade indicated that *arn* was lost during the evolution of *Enterobacter.*

Heteroresistance levels correlate with the intrinsic level of lipid A L-Ara4N-modification

To define the nature of structural modifications associated with colistin resistance we extracted lipid A from various isolates of *E. xiangfangensis* grown either in presence or absence of colistin (2 mg/L) (Figure 2a, Supplementary data 4). Only isolates encoding *arn* (most L-1 isolates) could modify their lipid A with L-Ara4N. Such isolates grown in the absence of colistin showed lipid A molecules with only a single L-Ara4N substitution either at the 4'- (R1) or 1'- (R2) phosphate. Growth of these isolates in presence of colistin resulted in a significant overall increase of L-Ara4N modifications, and in part with double substitution. Strains that lacked the *arn* gene cassette did not show any modifications of lipid A. The general structure of *Enterobacter* lipid A is shown in Figure 2b, all lipid A species observed in our MS analyses are listed in Supplementary data 4. Representative MS spectra for *E. xiangfangensis* isolates are shown in Supplementary Figure 3 and such spectra are the source of reported ion intensities used for lipid A ratio calculation (Supplementary data 4). Of note, in isolates carrying the *mcr-9* gene, no phosphoethanolamine (PEtN) modification was observed, indicating that *mcr-9* is either non-functional or not induced under the growth conditions used (Supplementary data 4). The same MS-based lipid A analysis was performed for representative strains of the remaining 11 *Enterobacter* species grown in colistin-free media. The varying levels of L-Ara4N modification of lipid A detected under these culture conditions was found to be species-dependent (Figure 2c, Supplementary data 4).

We wondered if the levels of lipid A modification seen in cultures grown in the absence of colistin reflected the fraction of heteroresistant bacteria observed by the PAP assay. In addition, we compared HRFs to the levels of lipid A modified with L-Ara4N for representative strains of all the species studied when grown under the same conditions. We found a very strong positive correlation between the proportions of L-Ara4N-modified lipid A and their HRF (Pearson correlation coefficient: 0.90 and 0.72 for 8 and 32 mg/L colistin, respectively) indicating heteroresistance essentially reflects the level of intrinsically modified lipid A when isolates are grown in media without colistin (Figure 2d, Supplementary Table 4).

We next determined if L-Ara4N-modification levels of lipid A represented minor sub-populations within isogenic growing population. To differentiate between intact and damaged membranes from isogenic growing population, we used LIVE/DEAD^{TM} staining following exposure of bacteria to a high concentration (64 mg/L) of colistin for 15 min and observed cell populations using fluorescence microscopy. Analysis of *arn* carrying *Enterobacter* species revealed sub-populations of cells with intact membranes, inferring their survival during colistin treatment (Figure 2d, Supplementary Figure 4). The percentage of cells survived showed positive correlation (r=0.89) with the levels of modified lipid A. Exposure of bacteria for longer time (up to 4 hours) at colistin concentration (64 mg/L) did not result in additional killing and percentage of cells that survived were comparable to that of 15 min. The high correlation of lipid A L-Ara4N-substitution levels to HRF and proportions of live cells under identical growth conditions, suggests that a genetic basis underlies the presence of sub-populations observed for the different species.

Genetic determinants of colistin resistance and heteroresistance

In *Enterobacterales*, expression of *arn* has been shown to be dependent on *pmrAB, phoPQ*, and the PhoPQ-feedback inhibitor *mgrB*[57,58]. To determine the role of genetic components

involved in the colistin resistance or heteroresistance, we created and studied isogenic mutants of *arn* (i.e., *arnBCADTEF* gene cassette), *pmrAB, phoPQ*, and *mgrB* in a representative *E. xiangfangensis* L-1 isolate RBK-17-0394-1 (henceforth called Exf394).

While the parental isolate Exf394 exhibited a MIC of 2-32 mg/L towards colistin, the Δ*arn* mutant had a MIC of only 0.5 mg/L (Figure 3). Reintroduction of the *arn* on the low copy pBBR1MCS-2 plasmid reinstated the MIC to between 8-16 mg/L, demonstrating an absolute requirement of this gene cassette for colistin resistance activity. Deletion of *phoPQ* also lowered colistin resistance (2 mg/L), while the MIC exhibited by the Δ*pmrAB* mutant was comparable to that of the parental strain at 4-16 mg/L. For the Δ*mgrB* mutant, encoding the negative regulator of PhoPQ, high MIC levels of 128->512 mg/L were observed. Genetic complementation by reintroduction of the respective gene deleted in these mutants, restored MIC levels to that seen with the parental Exf394 strain.

We extended this data by examining the levels of L-Ara4N substitution of lipid A. Lipid A preparations from the Δ*arn* and Δ*phoPQ* mutants were totally devoid of L-Ara4N modifications, while the Δ*pmrAB* and the parental strain Exf394 showed similar levels of lipid A substitutions under both growth conditions (Supplementary data 4, Supplementary Table 5). The Δ*mgrB* mutant exhibited high levels of L-Ara4N substitution even when grown in the absence of colistin. In the LIVE/DEAD^{TM} staining assay the Δ*mgrB* mutant showed higher numbers of viable bacteria as compared to WT, while Δ*arn* and Δ*phoPQ* had relatively lower numbers of surviving bacteria following colistin treatment (Supplementary Figure 5).

Allelic differences in *phoQ* and *mgrB* determines colistin resistance and heteroresistance

To understand variations in the MICs and HRFs of different *Enterobacter* species we examined *phoPQ/mgrB* loci for allelic differences. Analysis of nucleotide sequences of gene and their promoter regions showed high conservation for the *mgrB* locus (Supplementary data 6). Significant differences were noted within the coding regions of PhoP and PhoQ, while the respective *phoPQ* promoter region was highly conserved. Species-wise differences were observed in the amino acid sequences of PhoPQ suggesting allelic changes could lead to differences in resistance and heteroresistance levels.

As the various *Enterobacter* species that harbor different alleles of PhoPQ have a wide range of MICs and HRF, we introduced the *phoPQ* alleles from *E. bugandensis* RBG-17-0246-1 (Ebg246) and *E. roggenkampii* RPB-17-0516-2 (Erg516), that each exhibit MICs of between 64->128 mg/L, into the Exf394Δ*phoPQ* mutant (Figure 4). Complementation with the heterologous $phoPQ_{246}$ or $phoPQ_{516}$ genes in Δ$phoPQ_{394}$ mutant resulted in recombinants that exhibited MICs of 64-128 mg/L, respectively. The HRF increased to 42.33±4.78% and 10.83±1.43% on 8 and 32 mg/L colistin with $phoPQ_{246}$ complementation. For the strain complemented with $phoPQ_{516}$, HRFs were 7.13±2.75% and 1.05±0.19% on 8 and 32 mg/L colistin, respectively (Supplementary Table 5).

These results were also examined by analysis of transcript levels of the *arnBCADTEF* operon. Compared to complementation with $phoPQ_{394}$, the transcript levels of *arnBCADTEF* increased by 20.84-fold when complemented with $phoPQ_{246}$ and 2.75-fold with the $phoPQ_{516}$ allele following transfer into the Exf394 *ΔphoPQ* mutant (Supplementary Figure 6). Mass spectrometric analysis of L-Ara4N modifications of lipid A indicated that it was highest in *ΔphoPQ* strain complemented with $phoPQ_{246}$ followed by $phoPQ_{516}$ and then $phoPQ_{394}$. This was further

validated using the LIVE/DEAD assay. We observed higher ratios of viable bacteria for the *phoPQ*$_{246}$ complemented strain, followed by *phoPQ*$_{516}$ and the *phoPQ*$_{394}$ complemented strains.

To differentiate between the individual role of alleles of *phoP* and *phoQ*, we created hybrid versions of *phoPQ* by generating chimeric combinations of *phoP* and *phoQ* from each of Exf394, Ebg246, and Erg516, and transferring them to the Δ*phoPQ*$_{394}$ mutant (Figure 4). Among the different combinations of *phoP* and *phoQ* alleles, those that carried *phoQ*$_{246}$ had the highest MICs and HRF levels, followed by *phoQ*$_{516}$, and was lowest with *phoQ*$_{394}$. Unlike the results obtained with the respective *phoQ* alleles, no significant difference in MICs was observed with *phoP* alleles deriving from the 246/516/394 strains. These data implicate the PhoQ sensor component of the PhoPQ two-component system as a major determinant in the activation of *arnBCADTEF* transcription and expression.

We next addressed the contribution of the *mgrB* gene from Ebg246/Erg516, whose sequences are identical, through introduction of the complementing plasmids into the Δ*mgrB*$_{394}$ mutant. Recombinants complemented with the *mgrB*$_{246/516}$ allele exhibited an elevated MIC of 64 mg/L, with a HRF value of 2.1±1.13% and 0.96±0.66% at 8 and 32 mg/L of colistin. In contrast, complementation with the homologous *mgrB*$_{394}$ allele reduced colistin MIC values to 4-16 mg/L and expressed a HRF value of 0.34±0.24% and 0.22±0.09% at 8 and 32 mg/L of colistin, respectively (Figure 4).

An assay for unambiguous determination of colistin resistance in *Enterobacter* spp.

For the species *Salmonella typhimurium* and *Escherichia coli*, the two-component system (TCS) PhoPQ senses acidic pH as an environmental signal and regulates components required for the adaptation and survival of bacteria under these conditions. We reasoned that screening of

Enterobacter isolates grown in culture media at a low pH would overcome MgrB-mediated suppression of PhoQ and lead to constitutive expression of the *arn* gene cassette, thereby promoting colistin resistance. We examined all the isolates in this study for colistin resistance by growing cultures at pH 7 and pH 5. Those *Enterobacter* isolates that carry intact copies of genes corresponding to the *phoPQ/mgrB/arn* axis, showed 4 to 16-fold higher MICs when tested at pH 5 and generally in excess of 256 mg/L (Figure 5a, Supplementary data 1). The PAP test carried out by growing Exf394 on colistin LB agar plate adjusted for pH 5 showed a higher HRF (35.41±7.78% and 5.33±0.29%, on 8 and 32 mg/L colistin, respectively) than that at pH 7 (0.745±0.27% and 0.505±0.15%) (Figure 5b). Mass spectrometric analysis of Exf394 grown at pH 5 and pH 7 validated these results and showed higher L-4AraN-modification levels of lipid A in isolates grown at pH 5 as compared to pH 7. On the other hand, at pH 9, the Exf394 was completely sensitive, and lipid A modifications were lower as compared to pH 9. Thus, at pH 5 suppression of PhoQ by MgrB is overcome, and results in the activation of the PhoPQ-dependent regulon that includes the *arnBCADTEF* gene cassette.

Discussion

Taxonomic uncertainty associated with identifying members of the ECC together with problems relating to routine microbiology susceptibility testing for colistin resistance underestimate both the true prevalence of these bacteria in causing infections and mask the extent of how widespread this resistance is in these bacteria. ECC isolates, along with *E. coli* and *Klebsiella pneumoniae*, often harbor carbapenemases, forcing the therapeutic use of colistin worldwide. An expansion of genomic lineages of colistin-resistant *Enterobacter* species would be potentially concerning as relatively few antibiotic combinations remain as further treatment

options. Adding to these concerns, problems associated with heteroresistance pose a serious threat because infections with *Enterobacter* are notoriously persistent and difficult to treat. The development of simple, reliable diagnostic methods that recognize resistance and heteroresistance is therefore urgently needed.

Genome-based taxonomy provided consistent and robust topologies allowing the identification of *E. xiangfangensis* as the predominant clinical species both in Germany as well as worldwide. To improve the utility of this taxonomic scheme and further encourage studies on historical isolates, we carried out a backward compatibility analysis to the previously developed based on *hsp60* gene analysis[36]. This enabled retrospective analysis of *Enterobacter* isolates from several studies published previously, e.g. Hoffman et al. (2003; comprising isolates from both the USA and European countries)[36], Paauw et al. (2008, The Netherlands)[7], Morand et al. (2009, France)[59], Garinet et al. (2018, France)[59], Moradigaravand et al. (2016, UK)[60], Akbari et al. (2016, Iran)[61], Peirano et al. (2018; carbapenemase resistant worldwide isolates)[12], Wang et al. and Zhou et al. (2018, China)[62,63]. From these retrospective studies we found a predominance of *hsp60* clusters -III, -VI, and particularly -VIII, which are essentially identical to lineage-I isolates of *E. xiangfangensis* determined here, suggesting that this is the most commonly occurring species among clinical isolates worldwide, going back over a period of at least two decades (Supplementary data 2).

Although heteroresistance towards colistin in *Enterobacter* species was first reported in 2007[64], the often inconsistent MIC values and complications in interpreting data from the "skipped well phenomenon" have made determinations on the overall prevalence of colistin resistance difficult[44,58,65,66]. Thus, according to current WHO classifications, *Enterobacter* species are listed as being generally susceptible to colistin[15,16]. The emergence of transferable colistin resistance

518 through plasmids, and in particular the extremely high occurrence of *mcr-9* (~20%) in
519 *Enterobacter* spp. had added further urgency in understanding the true levels of colistin resistance
520 in ECC[67].

521 Levels of colistin resistance depend on the extent of modified lipid A molecules with
522 substitutions that reduce the electronegative potential of lipopolysaccharide (LPS). From this study
523 we conclude that L-Ara4N modification of lipid A is solely responsible for colistin resistance (and
524 heteroresistance) in ECC, as we did not observe any P-EtN-based substitutions, even in those
525 isolates that carry *mcr-9* but lack the *arnBCADTEF* gene cassette. Based on the taxonomic scheme
526 developed and from functional characterization of lipid A modifications in representative isolates
527 we conclude that 20 of 23 *Enterobacter* species are inherently colistin resistant. Interestingly for
528 the remaining species, where most isolates are members of the sublineages L-2, L-3, and L-4 of *E.*
529 *xiangfangensis*, we show clonal loss of the *arnBCADTEF* gene cassette associated with a clean
530 excision event. As these represent a significant subset of *Enterobacter* isolates found at different
531 sites in this study, the implication of this observation, i.e., if it is adaptive or provides growth
532 advantages needs to be explored. Alternatively, the ubiquitous presence of this gene cassette in
533 several members of the order *Enterobacterales* including *Enterobacter*, *E. coli*, and *K. pneumoniae*
534 suggest that in these lineages, it could be rapidly reacquired from close relatives.

535 The species-specific heteroresistance observed in this study and in previous studies of
536 Guerin et al.[44], who correlated their findings with the *hsp60* clustering scheme, showed that the
537 degree of heteroresistance differ across *Enterobacter* species (Table 2). In *Enterobacterales*,
538 resistance towards colistin is complex and multifactorial requiring genes encoding structural
539 components as well as those involved in LPS transport and its modification, together with
540 associated regulatory genes[68]. Quantitative lipid A analysis of isogenic mutants and

complementation analysis demonstrated that colistin resistance requires the presence of an intact *arn/phoPQ/mgrB* axis. Strikingly, when complemented, recombinants also exhibited skip-well phenomenon in MIC testing assays, indicating that these genes also control inherent heteroresistance capacity[56,69].

A major finding in this study using heterologous complementation assays, was that those allelic differences in the two component *phoPQ* sensor-activator system from different species govern levels of heteroresistance. Thus, isolates that exhibit high heteroresistance levels could confer this property, when the respective *phoPQ* alleles were transferred to an isogenic mutant strain from an isolate exhibiting low heteroresistance propensity. Quantitative transcriptional analysis demonstrated overexpression of the *arnT* operon in the isogenic mutant depending on the *phoPQ* allele introduced. This increased transcription also corresponded to an increase in the level of L-4AraN modification of lipid A in the complemented recombinant strains. By creating chimeric *phoPQ* allelic pairs, we demonstrated that qualitative changes in the periplasmic membrane interacting domain of the sensor PhoQ control expression levels of the *arnBCADTEF* gene cassette. Using a similar approach, we determined that the level of colistin resistance is also determined by allelic differences in its inhibitor peptide MgrB.

Our observations are supported by molecular modelling studies. The PhoQ-MgrB interactions predicted that introduction of the altered $mgrB_{246/516}$ allele from a highly resistant strain would significantly alter the MIC level of the moderately colistin resistant strain Exf394 because of the higher activation energy required for its interaction with the $phoPQ_{394}$ allele (Supplementary Figure 7). As predicted, introduction of $mgrB_{246/516}$ into the Exf394Δ*mgrB* enhanced its MIC suggesting less efficient repression of the endogenous PhoQ sensor component by this variant. Detailed analysis of PhoQ-MgrB indicated that the α/β-core of PhoQ (aa 43 -180) interacts with

564 the C-terminal aa residues 34-47 of MgrB. Our data extends data from a recent study showing the
565 importance of the interaction of the C-terminal periplasmic region of MgrB with PhoQ.

566 Previous studies showed that two pathways leading to acquired colistin resistance may
567 exist in various *Enterobacter* and *Klebsiella* species, one involving the PmrAB, and another
568 associated with PhoPQ[68]. Our data are consistent with the role of the TCS PhoPQ and its inhibitor
569 MgrB, but do not support a role for the PmrAB pathway. This is consistent with data presented by
570 Kang et al.[58] for *E. cloacae*, who did not find the role of *pmrAB* in colistin resistance. It was
571 previously shown that colistin-resistant sub-populations of an isolate of *E. cloacae* increased in
572 peritoneally infected mice even in the absence of co-treatment with colistin, and that this property
573 was associated with the phagocytic capacity of resident macrophages[65]. This selective increase in
574 the frequencies of these subpopulations is attributed to components of the innate immune response
575 including cationic antimicrobial peptides, reactive oxygen species generated by NADPH oxidases
576 and lysozyme[65].

577 We show here that these sub-populations are already present during growth in broth
578 cultures without antibiotics suggesting that a fraction of an isogenic population of bacteria
579 experience microenvironment changes that results in metabolic heterogeneity[70–72]. These includes
580 changes in osmolarity, oxygen tension, ion flux, pH, as well as periplasmic transmembrane
581 proteins that are all monitored by periplasmic histidine kinase sensors, such as PhoQ. In the case
582 of *S. typhimurium* and other related bacteria, activation of the PhoQ sensor by antimicrobial
583 peptides and acidic pH are separate and additive[73,74]. Our studies suggest a preeminent role for
584 environmental pH as a signal since cells pre-grown at acidic pH exhibited higher MICs and HRFs,
585 which is further elevated when cells are pre-grown at acidic pH and in presence of colistin. Under
586 conditions of growth at physiological pH, heteroresistance is a function of the qualitative

differences in protein interactions that govern PhoPQ-MgrB interactions in sub-populations sensing microenvironmental pH differences. Flooding the growth environment with protons would constitutively activate PhoPQ and as expected, prior exposure of bacterial cells in the absence of colistin at pH 5 induced high levels of lipid A L-Ara4N-modification and exhibited elevated levels of HRF as compared to those at pH 7 (Figure 6). Both acidic pH and antimicrobial peptides are likely to be encountered in the host viz., during passage of the gastrointestinal tract or within the phagolysosomes of macrophages following phagocytosis and sensing of these signals would activate transitory gene expression programs to enable bacterial resilience in responding to the varying environments encountered during infection.

Our data establish a comprehensive overview of the major *Enterobacter* species that are associated with BSI worldwide. The generation of species-specific profiles – using MALDI-TOF, based on the taxonomic scheme described here – is highly desirable and would provide for rapid and robust species designations in clinical microbiological laboratories. We show that members of the species *Enterobacter* are inherently colistin-resistant due to L-Ara4N modification of lipid A. Finally, we identify environmental fluctuations in pH as the major determinant of heteroresistance levels via sensing by the PhoPQ two component regulatory system and its inhibitory MgrB peptide and exploit this information to develop a simple, robust assay that unambiguously monitors colistin resistance in *Enterobacter* spp. and would avoid treatment failures in healthcare institutions worldwide. The elements driving clonal heteroresistance are also highly conserved in the order Enterobacterales, such as in the species *Klebsiella*, thereby opening the path for generic tests to accurately determine colistin resistance across a broad spectrum of clinically relevant bacteria.

References:

1. Murray, C. J. *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**, 629–655 (2022).

2. Kern, W. V. & Rieg, S. Burden of bacterial bloodstream infection—a brief update on epidemiology and significance of multidrug-resistant pathogens. *Clinical Microbiology and Infection* **26**, 151–157 (2020).

3. Thaden, J. T. *et al.* Increased costs associated with bloodstream infections caused by multidrug-resistant gram-negative bacteria are due primarily to patients with hospital-acquired infections. *Antimicrob. Agents Chemother.* **61**, (2017).

4. Diekema, D. J. *et al.* The microbiology of bloodstream infection: 20-year trends from the SENTRY antimicrobial surveillance program. *Antimicrob. Agents Chemother.* **63**, (2019).

5. Diekema, D. J., Pfaller, M. A., Jones, R. N. & Beach, M. Survey of Bloodstream Infections Due to Gram-Negative Bacilli: Frequency of Occurrence and Antimicrobial Susceptibility of Isolates Collected in the United States, Canada, and Latin America for the SENTRY Antimicrobial Surveillance Program, 1997. *Clin. Infect. Dis.* **29**, 595–607 (1999).

6. Tacconelli, E. *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* **18**, 318–327 (2018).

7. Paauw, A. *et al.* Genomic Diversity within the Enterobacter cloacae Complex. *PLoS One* **3**, e3018 (2008).

8. Grimont, F. & Grimont, P. A. D. The Genus Enterobacter. in *The Prokaryotes* 197–214 (Springer New York, 2006). doi:10.1007/0-387-30746-X_9

9. Davin-Regli, A., Lavigne, J.-P. & Pagès, J.-M. Enterobacter spp.: Update on Taxonomy, Clinical Aspects, and Emerging Antimicrobial Resistance. *Clin. Microbiol. Rev.* (2019). doi:10.1128/cmr.00002-19

10. Nation, R. L. & Li, J. Colistin in the 21st century. *Curr. Opin. Infect. Dis.* **22**, 535–43 (2009).

11. Chavda, K. D. *et al.* Comprehensive Genome Analysis of Carbapenemase-Producing Enterobacter spp.: New Insights into Phylogeny, Population Structure, and Resistance Mechanisms. *MBio* **7**, e02093-16 (2016).

12. Peirano, G. *et al.* Genomic Epidemiology of Global Carbapenemase-Producing *Enterobacter* spp., 2008–2014. *Emerg. Infect. Dis.* **24**, 1010–1019 (2018).

13. FF, A., D, S., A, R. & C, P.-V. Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges. *Microorganisms* **8**, 1–12 (2020).

14. Hindler, J. A. & Humphries, R. M. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant gram-negative bacilli. *J. Clin. Microbiol.* **51**, 1678–1684 (2013).

15. WHO. *Global Antimicrobial Resistance Surveillance System (GLASS)*. (2018).

16. WHO. *Global Antimicrobial Resistance Surveillance System (GLASS): The detection and reporting of colistin resistance. Second edition*. (2021).

17. Mushtaq, S. *et al.* Inherent colistin resistance in genogroups of the Enterobacter cloacae complex: epidemiological, genetic and biochemical analysis from the BSAC Resistance Surveillance Programme. *J. Antimicrob. Chemother.* **75**, 2452–2461 (2020).

18. Gales, A. C., Jones, R. N. & Sader, H. S. Global assessment of the antimicrobial activity of polymyxin B against 54 731 clinical isolates of Gram-negative bacilli: report from the SENTRY

651 antimicrobial surveillance programme (2001–2004). *Clin. Microbiol. Infect.* **12**, 315–321 (2006).

652 19. Andersson, D. I., Nicoloff, H. & Hjort, K. Mechanisms and clinical relevance of bacterial
653 heteroresistance. *Nat. Rev. Microbiol.* **17**, 479–496 (2019).

654 20. Band, V. I. & Weiss, D. S. Heteroresistance: A cause of unexplained antibiotic treatment failure?
655 *PLOS Pathog.* **15**, e1007726 (2019).

656 21. CLSI-EUCAST. Recommendations for MIC determination of colistin (polymyxin E) As
657 recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. (2016).
658 Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/
659 General_documents/Recommendations_for_MIC_determination_of _colistin_March_2016.pdf.

660 22. Falgenhauer, J. *et al.* Whole-Genome Sequences of Clinical *Enterobacter bugandensis* Isolates
661 from Germany. *Microbiol. Resour. Announc.* **8**, (2019).

662 23. Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. Unicycler: Resolving bacterial genome
663 assemblies from short and long sequencing reads. *PLoS Comput. Biol.* (2017).
664 doi:10.1371/journal.pcbi.1005595

665 24. Chun, J. *et al.* Proposed minimal standards for the use of genome data for the taxonomy of
666 prokaryotes. *Int. J. Syst. Evol. Microbiol.* **68**, 461–466 (2018).

667 25. Wu, W., Feng, Y. & Zong, Z. Precise Species Identification for Enterobacter: a Genome
668 Sequence-Based Study with Reporting of Two Novel Species, Enterobacter quasiroggenkampii
669 sp. nov. and Enterobacter quasimori sp. nov. *mSystems* **5**, (2020).

670 26. Cho, G. S. *et al.* Polyphasic study of antibiotic-resistant enterobacteria isolated from fresh
671 produce in Germany and description of Enterobacter vonholyi sp. nov. isolated from marjoram
672 and Enterobacter dykesii sp. nov. isolated from mung bean sprout. *Syst. Appl. Microbiol.* **44**,

126174 (2021).

27. Richter, M. *et al.* Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci.* **106**, 19126–19131 (2009).

28. Figueras, M. J., Beaz-Hidalgo, R., Hossain, M. J. & Liles, M. R. Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. *Genome Announc.* (2014). doi:10.1128/genomeA.00927-14

29. Meier-Kolthoff, J. P., Auch, A. F., Klenk, H.-P. & Göker, M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* **14**, 60 (2013).

30. Page, A. J. *et al.* Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, 3691–3 (2015).

31. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313 (2014).

32. Treangen, T. J., Ondov, B. D., Koren, S. & Phillippy, A. M. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* **15**, 524 (2014).

33. Gardner, S. N., Slezak, T. & Hall, B. G. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. *Bioinformatics* **31**, (2015).

34. Tonkin-Hill, G., Lees, J. A., Bentley, S. D., Frost, S. D. W. & Corander, J. RhierBAPs: An R implementation of the population clustering algorithm hierbaps [version 1; referees: 2 approved]. *Wellcome Open Res.* **3**, 93 (2018).

694 35. Brynildsrud, O., Bohlin, J., Scheffer, L. & Eldholm, V. Rapid scoring of genes in microbial pan-
695 genome-wide association studies with Scoary. *Genome Biol.* **17**, 238 (2016).

696 36. Hoffmann, H. & Roggenkamp, A. Population genetics of the nomenspecies Enterobacter cloacae.
697 *Appl. Environ. Microbiol.* **69**, 5306–18 (2003).

698 37. Ondov, B. D. *et al.* Mash: fast genome and metagenome distance estimation using MinHash.
699 *Genome Biol.* **17**, 132 (2016).

700 38. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments.
701 *Nucleic Acids Res.* **47**, W256–W259 (2019).

702 39. Sullivan, M. J., Petty, N. K. & Beatson, S. A. Easyfig: a genome comparison visualizer.
703 *Bioinformatics* **27**, 1009–1010 (2011).

704 40. Carattoli, A. *et al. In Silico* Detection and Typing of Plasmids using PlasmidFinder and Plasmid
705 Multilocus Sequence Typing. *Antimicrob. Agents Chemother.* **58**, 3895–3903 (2014).

706 41. Galata, V., Fehlmann, T., Backes, C. & Keller, A. PLSDB: A resource of complete bacterial
707 plasmids. *Nucleic Acids Res.* (2019). doi:10.1093/nar/gky1050

708 42. Buchfink, B., Xie, C. & Huson, D. H. Fast and sensitive protein alignment using DIAMOND.
709 *Nat. Methods* **12**, 59–60 (2015).

710 43. Jia, B. *et al.* CARD 2017: expansion and model-centric curation of the comprehensive antibiotic
711 resistance database. *Nucleic Acids Res.* **45**, D566–D573 (2017).

712 44. Guérin, F. *et al.* Cluster-dependent colistin hetero-resistance in Enterobacter cloacae complex. *J.*
713 *Antimicrob. Chemother.* **71**, 3058–3061 (2016).

714 45. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli

K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* (2000). doi:10.1073/pnas.120163297

46. Näsvall, J., Knöppel, A. & Andersson, D. I. Duplication-Insertion Recombineering: A fast and scar-free method for efficient transfer of multiple mutations in bacteria. *Nucleic Acids Res.* (2017). doi:10.1093/nar/gkw1078

47. El Hamidi, A., Tirsoaga, A., Novikov, A., Hussein, A. & Caroff, M. Microextraction of bacterial lipid A: Easy and rapid method for mass spectrometric characterization. *J. Lipid Res.* **46**, 1773–1778 (2005).

48. Herzog, R. *et al.* A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. *Genome Biol.* (2011). doi:10.1186/gb-2011-12-1-r8

49. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* **29**, e45 (2001).

50. Molnar, K. S. *et al.* Cys-scanning disulfide crosslinking and bayesian modeling probe the transmembrane signaling mechanism of the histidine kinase, PhoQ. *Structure* **22**, 1239–1251 (2014).

51. Waterhouse, A. *et al.* SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* **46**, W296–W303 (2018).

52. Senior, A. W. *et al.* Improved protein structure prediction using potentials from deep learning. *Nat. 2020 5777792* **577**, 706–710 (2020).

53. Laskowski, R. A., MacArthur, M. W., Moss, D. S., Thornton, J. M. & IUCr. PROCHECK: a program to check the stereochemical quality of protein structures. *urn:issn:0021-8898* **26**, 283–291 (1993).

54. Dominguez, C., Boelens, R. & Bonvin, A. M. J. J. HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. *J. Am. Chem. Soc.* **125**, 1731–1737 (2003).

55. Elbediwi, M. *et al.* Global burden of colistin-resistant bacteria: Mobilized colistin resistance genes study (1980–2018). *Microorganisms* **7**, 461 (2019).

56. El-Halfawy, O. M. & Valvano, M. A. Antimicrobial heteroresistance: An emerging field in need of clarity. *Clinical Microbiology Reviews* **28**, 191–207 (2015).

57. Lippa, A. M. & Goulian, M. Feedback Inhibition in the PhoQ/PhoP Signaling System by a Membrane Peptide. *PLoS Genet.* **5**, e1000788 (2009).

58. Kang, K. N. *et al.* Colistin heteroresistance in Enterobacter cloacae is regulated by PhoPQ-dependent 4-amino-4-deoxy-l-arabinose addition to lipid A. *Mol. Microbiol.* **111**, 1604–1616 (2019).

59. Morand, P. C. *et al.* Specific Distribution within the Enterobacter cloacae Complex of Strains Isolated from Infected Orthopedic Implants. *J. Clin. Microbiol.* **47**, 2489–2495 (2009).

60. Moradigaravand, D., Reuter, S., Martin, V., Peacock, S. J. & Parkhill, J. The dissemination of multidrug-resistant Enterobacter cloacae throughout the UK and Ireland. *Nat. Microbiol.* **1**, 16173 (2016).

61. Akbari, M., Bakhshi, B. & Najar Peerayeh, S. Particular Distribution of Enterobacter cloacae Strains Isolated from Urinary Tract Infection within Clonal Complexes. *Iran. Biomed. J.* **20**, 49–55 (2016).

62. Wang, Y. Q. *et al.* Rapid Identification and Subtyping of Enterobacter cloacae Clinical Isolates Using Peptide Mass Fingerprinting. *Biomed. Environ. Sci.* **31**, 48–56 (2018).

63. Zhou, K. *et al.* Characterization of the population structure, drug resistance mechanisms and plasmids of the community-associated Enterobacter cloacae complex in China. *J. Antimicrob. Chemother.* **73**, 66–76 (2018).

64. Lo-Ten-Foe, J. R., De Smet, A. M. G. A., Diederen, B. M. W., Kluytmans, J. A. J. W. & Van Keulen, P. H. J. Comparative evaluation of the VITEK 2, disk diffusion, etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant Enterobacter cloacae and Acinetobacter baumannii strains. *Antimicrob. Agents Chemother.* **51**, 3726–3730 (2007).

65. Band, V. I. *et al.* Antibiotic failure mediated by a resistant subpopulation in Enterobacter cloacae. *Nat. Microbiol.* (2016). doi:10.1038/nmicrobiol.2016.53

66. Band, C. I., Satola, V. I. & Smith, S. W. Colistin Heteroresistance Is Largely Undetected among Carbapenem-Resistant Enterobacterales in the United States OBSERVATION Clinical Science and Epidemiology Downloaded from. doi:10.1128/mBio.02881-20

67. Wang, R. *et al.* The global distribution and spread of the mobilized colistin resistance gene mcr-1. *Nat. Commun.* **9**, (2018).

68. Aghapour, Z. *et al.* Molecular mechanisms related to colistin resistance in Enterobacteriaceae. *Infect. Drug Resist.* **Volume 12**, 965–975 (2019).

69. Dewachter, L., Fauvart, M. & Michiels, J. Bacterial Heterogeneity and Antibiotic Survival: Understanding and Combatting Persistence and Heteroresistance. *Molecular Cell* **76**, 255–267 (2019).

70. Takhaveev, V. & Heinemann, M. Metabolic heterogeneity in clonal microbial populations. *Current Opinion in Microbiology* **45**, 30–38 (2018).

780 71. Henderson, I. R., Owen, P. & Nataro, J. P. *MicroReview Molecular switches ± the ON and OFF*
781 *of bacterial phase variation*.

782 72. Meouche, I. El, Siu, Y. & Dunlop, M. J. Stochastic expression of a multiple antibiotic resistance
783 activator confers transient resistance in single cells. *Nat. Publ. Gr.* (2015). doi:10.1038/srep19538

784 73. Prost, L. R. & Miller, S. I. The Salmonellae PhoQ sensor: mechanisms of detection of phagosome
785 signals. (2008). doi:10.1111/j.1462-5822.2007.01111.x

786 74. Bader, M. W. *et al.* Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell* **122**,
787 461–472 (2005).

788 75. Gu, C. T., Li, C. Y., Yang, L. J. & Huo, G. C. Enterobacter xiangfangensis sp. nov., isolated from
789 Chinese traditional sourdough, and reclassification of Enterobacter sacchari Zhu et al. 2013 as
790 Kosakonia sacchari comb. nov. *Int. J. Syst. Evol. Microbiol.* **64**, 2650–2656 (2014).

791 76. Richter, M. & Rossello-Mora, R. Shifting the genomic gold standard for the prokaryotic species
792 definition. *Proc. Natl. Acad. Sci.* **106**, 19126–19131 (2009).

793 77. Sutton, G. G., Brinkac, L. M., Clark, T. H. & Fouts, D. E. Enterobacter hormaechei subsp.
794 hoffmannii subsp. nov., Enterobacter hormaechei subsp. xiangfangensis comb. nov., Enterobacter
795 roggenkampii sp. nov., and Enterobacter muelleri is a later heterotypic synonym of Enterobacter
796 asburiae based on computational a. *F1000Research* **7**, 521 (2018).

797 78. Hoffmann, H. *et al.* Enterobacter hormaechei subsp. oharae subsp. nov., E. hormaechei subsp.
798 hormaechei comb. nov., and E. hormaechei subsp. steigerwaltii subsp. nov., three new subspecies
799 of clinical importance. *J. Clin. Microbiol.* **43**, 3297–3303 (2005).

800 79. Kieffer, N. *et al.* mcr-9, an inducible gene encoding an acquired phosphoethanolamine transferase
801 in Escherichia coli, and its origin. *Antimicrob. Agents Chemother.* (2019).

802 doi:10.1128/AAC.00965-19

803

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817 **Authors contributions:** S.D. and T.C. conceived research on colistin resistance/heteroresistance.
818 The clinical study was conceived and conducted by the R-Net study group. S.D. analyzed
819 genomes, performed the experiments together with R.F. and C.G.. N.G. and D.S. designed and
820 performed the mass spectrometry studies. S.D. and R.F. created mutants and performed
821 complementation analysis. B.V.K. carried out protein modeling studies and constructed models.
822 J.F. and C.I. collected isolates, AMR and other meta-data from partner sites. LF carried out the
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825 and relevant metadata for research from respective study sites. C.I., N.G., D.S., and T.C.

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Tables:

Table 1: Isolates encoding *arnBCDATEF* gene cassette exhibit colistin resistance and heteroresistance. A total of 165 isolates of different *Enterobacter* species were studied for their minimum inhibitory concentration (MIC) and representative isolates of each species were subject to population analysis profile (PAP) test. All the isolates carrying intact *phoPQ/mgrB/arnBCADTEF* genes exhibited MICs in the range of 2 to >512 mg/L indicating resistance. The MIC of individual isolates varied in triplicate and often exhibited the "skipped well phenomenon" suggesting heteroresistance. PAP analysis showed heteroresistance frequency (HRF, number of cells recovered on LB agar plate containing 8 or 32 mg/L of colistin / number of cells inoculated) of *Enterobacter* species to vary between none to 41.7%. *Enterobacter* species exhibit a different range of MICs and HRF. Data for individual isolates are presented in Supplementary data 1. key; *: 3 isolates of *E. xiangfangensis* (belonging to ST133) lacking *arnBCADETF* showed a MIC less than 2-4 mg/L, while a single isolate of *E. xiangfangensis* L-1 (RBL-17-0250-3) carried a truncated *phoQ* gene. $: the single isolate of *E. vonholyi* observed in this study also carried a truncated *phoQ* gene.

***Enterobacter* species (OGRI)**	***arnBCADTEF* gene cassette**	**Colistin resistance**	**Colistin heteroresistance**
E. asburie	+	+	+
E. bugandensis	+	+	+
E. cancerogenus	+	+	+
E. mori	+	+	+
E. chengduensis	+	+	+
E. roggenkampii	+	+	+
E. vonholyi $	- $	- $	- $
E. cloacae	+	+	+
E. kobei	+	+	+
E. ludwigii	+	+	+
E. xiangfangensis L-I *	+*	+*	+*
E. xiangfangensis L-II	-	-	-
E. xiangfangensis L-III	-	-	-
E. xiangfangensis L-IV	-	-	-
E. wuhouensis	-	-	-

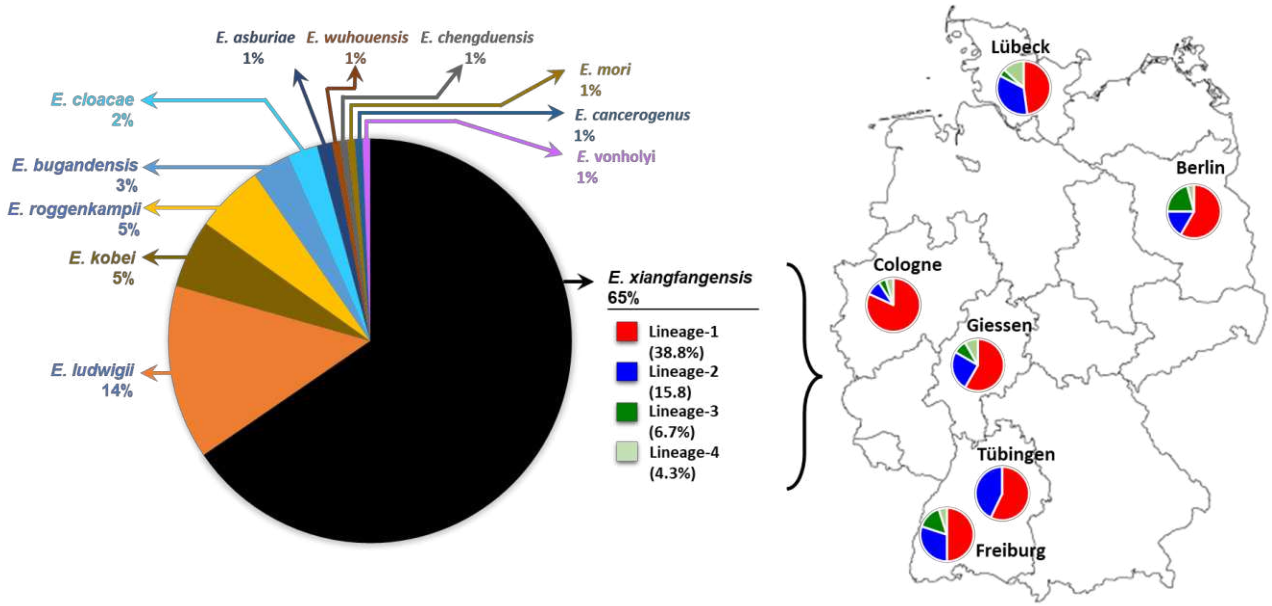
Table 2: Colistin resistance and heteroresistance of the *Enterobacter* species. A total of 165 isolates of different *Enterobacter* species were studied for their MIC and subject to PAP analysis. All the isolates carrying intact *phoPQ/mgrB/arnBCADTEF* genes exhibited MICs in the range of 2 to >512 mg/ml. PAP analysis showed heteroresistance frequency of *Enterobacter* species to vary between none to 48.4%. Different *Enterobacter* species exhibit different range of MICs and HRF. Data for individual isolates are presented in Supplementary data 1b. key; *: 3 isolates of *E. xiangfangensis* (belonging to ST133) lack *arnBCADETF*. $: a single isolate of *E. xiangfangensis* L-1 (RBL-17-0250-3) and a single isolate of *E. vonholyi* carried truncated phoQ gene. (+: present, -: absent)

Enterobacter species (OGRI)	hsp60 cluster	arnBCADTEF gene cassette	Colistin resistance / heteroresistance	Colistin MIC range by BMD (mg/L)	Heteroresistant frequency by PAP (%)	
					8 mg/L	32 mg/L
E. asburie	I	+	+	128-256	00.44 - 05.30	00.11 - 1.20
E. bugandensis	IX	+	+	64->512	04.10 - 25.80	01.63 - 05.28
E. cancerogenus	XIII	+	+	64-128	00.75 - 01.78	00.24 - 02.54
E. mori		+	+	32-64	00.96 - 01.44	00.53 - 00.55
E. chengduensis	IV	+	+	64-128	10.35 - 14.95	06.73 - 09.05
E. roggenkampii		+	+	64->512	00.00 - 09.70	00.00 - 05.38
E. vonholyi $		-$	-$	2-4	0	0
E. cloacae	XI	+	+	512->512	00.00 - 48.38	00.00 - 24.09
	XII	+	+			
E. kobei	II	+	+	8->512	00.00 - 01.19	00.00 - 00.55
E. ludwigii	V	+	+	4-256	00.00 - 01.07	00.00 - 00.14
E. xiangfangensis L-I *	VIII	+*	+*	2-128	00.00 - 00.75	00.0 - 00.50
E. xiangfangensis L-II	III	-	-	2-4	0	0
E. xiangfangensis L-III	VI	-	-	2-4	0	0
E. *xiangfangensis* L-IV		-	-	2-4	0	0
E. wuhouensis	new	-	-	4-6	0	0

Figures

Figure 1: (a) Distribution of *Enterobacter* species among six university hospitals in Germany. A total of 165 *Enterobacter* isolates were studied. Genome-based species classification showed *E. xiangfangensis* as the most frequent species at all centers. Values in parentheses denote the % of total *Enterobacter* (n=165) isolates. **(b) Phylogenomic distribution of the *Enterobacter* isolates obtained in between 2011-2018 from six different study centers.** Whole genome sequence-based study of 165 *Enterobacter* isolates revealed 65.4% of isolates to be *E. xiangfangensis.* Bayesian Analysis of Population Structure (BAPS) in concordance with the multi-phylogenomic approach (concatenate core genes (shown here), core- and whole-genome) clustered *E. xiangfangensis* isolates into four lineages. A majority (60% of *E. xiangfangensis*) isolates belonged to Lineage-1, which harbor *arnBCADTEF* gene cassette associated with colistin heteroresistance. Importantly, 9 *E. xiangfangensis* and 4 other *Enterobacter* species isolates carried the recently identified mobile colistin resistance gene *mcr-9*. A total of seven isolates, all *E. xiangfangensis,* harboured genes for known carbapenemases. The MLST and hsp60 types were well correlated to the phylogenomic lineages. The bootstrap values are shown in blue next to respective nodes only for four lineages. **(c)** Worldwide map depicting countries in which *E. xiangfangensis*, particularly isolates of L-1, is predominant (61 to 100% of all *Enterobacter* species) (filled in red).

885 (a)



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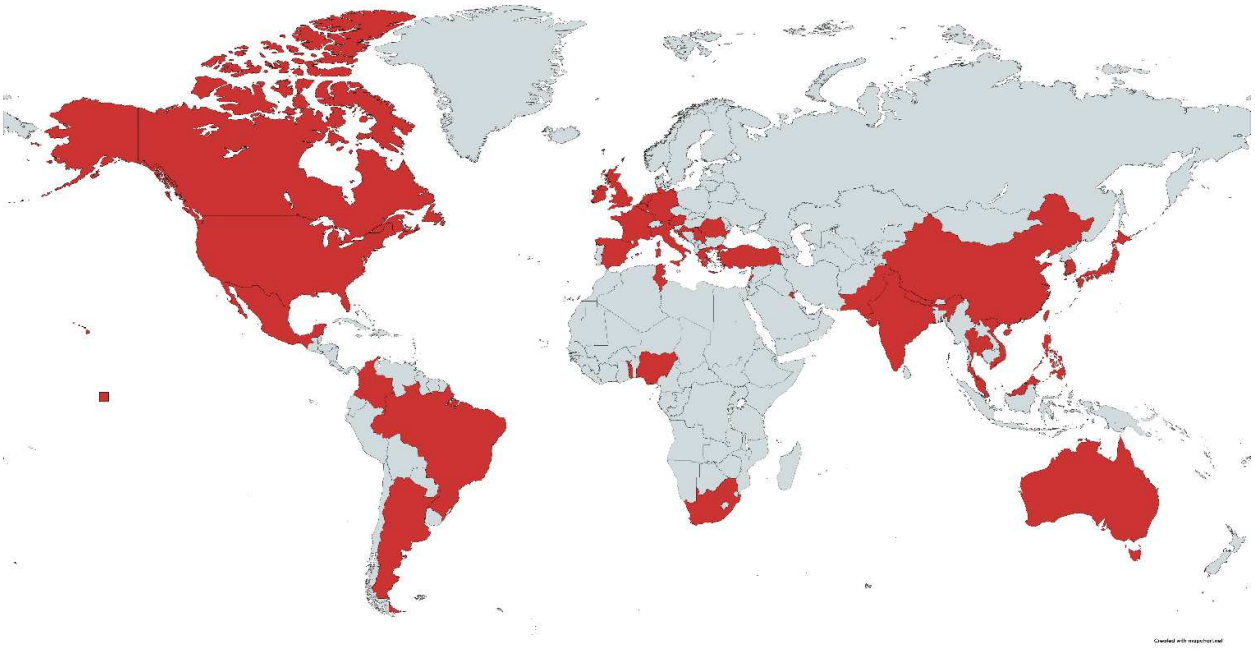
887 (b) next page

888 (c)



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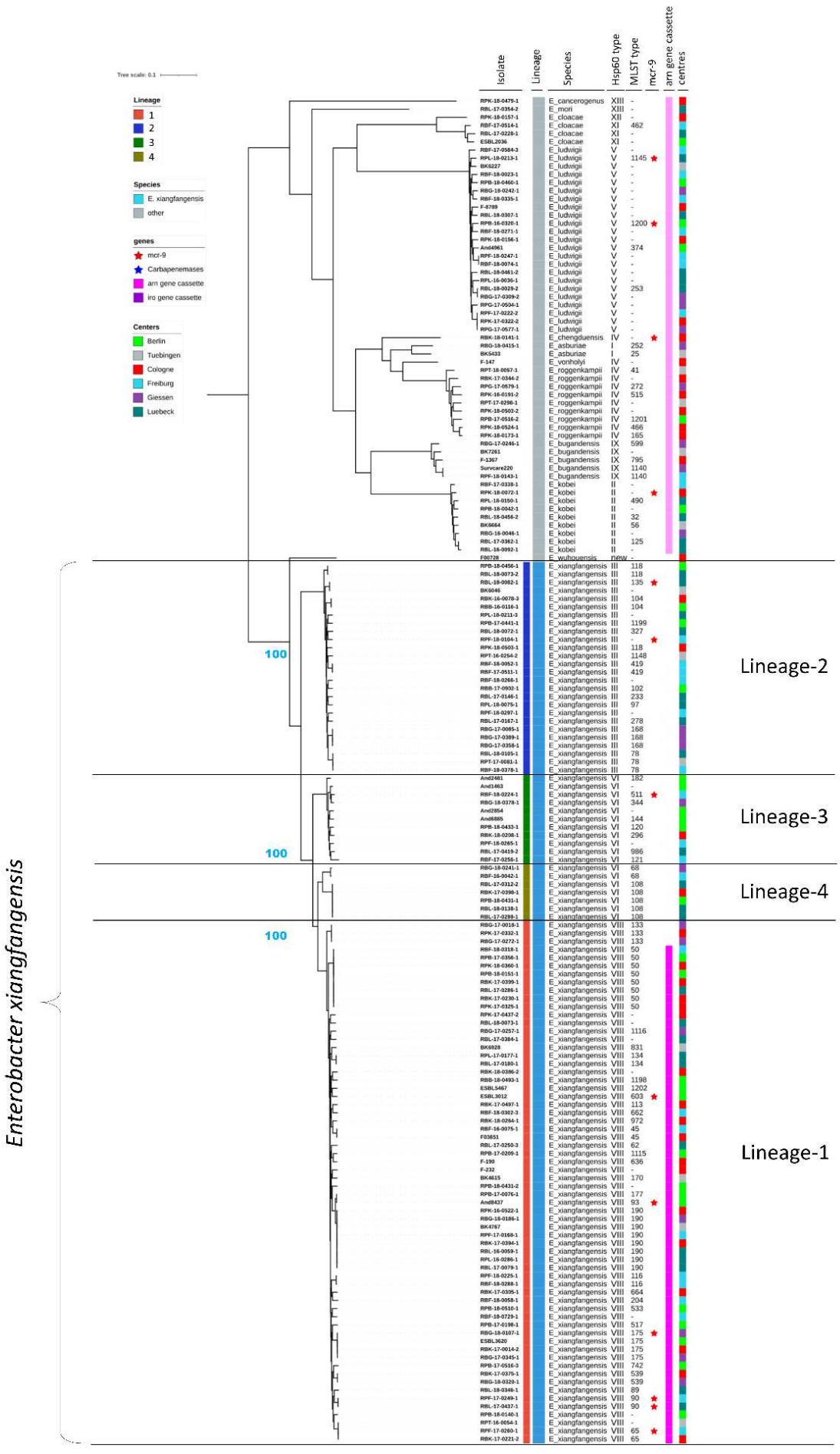
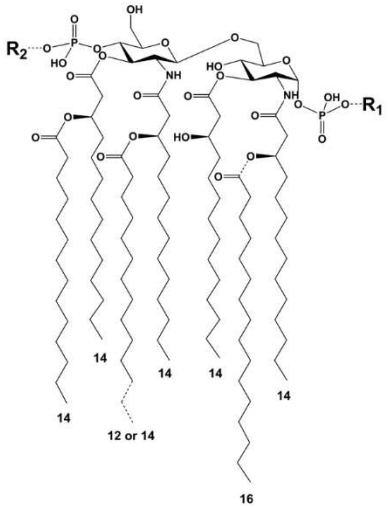

Tree scale: 0.1
Lineage
1
2
3
4
Species
E. xiangfangensis
other
genes
mcr-9
Carbapenemases
arn gene cassette
iro gene cassette
Centers
Berlin
Tuebingen
Cologne
Freiburg
Giessen
Luebeck
Isolate
Lineage
Species
Hsp60 type
MLST type
mcr-9
arn gene cassette
centres
100
100
100
Enterobacter xiangfangensis
Lineage-2
Lineage-3
Lineage-4
Lineage-1

Figure 2: Analysis of L-Ara4N-modification levels of lipid A and their correlation with heteroresistance frequency and number of cells survived after colistin treatment. (a) Heat map summarizing the MS-based analysis of L-Ara4N-modification levels of lipid A in selected *E. xiangfangensis* strains. Corresponding, representative mass spectra are shown in Supplementary Figures 3, further details are specified in Supplementary data 4. (**b)** General chemical structure of Enterobacter lipid A. **(c)** Heat map summarizing the MS-based analysis of L-Ara4N-modification levels of lipid A in 11 other *Enteorbcter* species observed in this study. (**d)** Various *Enterobacter* isolates grown at identical conditions (LB broth, $O.D._{600}$ 0.8-1.0, without colistin) were analyzed for L-Ara4N-modification of lipid A (see panel a), Supplementary data 4) and for their correlation with % of heteroresistant cells on plate containing 8 and 32 mg/L of colistin, and % of cells survived after 64 mg/L colistin treatment for 15 min. The % of heteroresistance CFUs at 8 and 32 mg/L was positively associated with L-Ara4N-modification levels of lipid A (Pearson's correlation coefficient, r = 0.90 and 0.72, respectively). This data suggested colistin heteroresistance to be directly associated with such levels. Given that only a fraction of isogenic growing population survives on plate containing 8 and 32 mg/L of colistin, we determine the % of cells that can survive after colistin treatment (64 mg/L for 15 min) by Live/Dead^{TM} staining assay. This assay showed a positive association (r = 0.89) between % of cell survived and L-Ara4N-modified lipid A. This data indicated that only certain number of cells from isogenic growing population carried modified lipid A and can survive when exposed to colistin, resulting in the heteroresistance phenomenon. This data show that cells with L-Ara4N-modified lipid A form the founder population of the colistin heteroresistance. (Also see Supplementary Table 1).

a

Species	Isolate	lineage	*mcr-9* gene	*arn* operon	MIC	Ara4N-substitution of lipid A species					
						no colistin			colistin (2 mg/L)		
						no subst.	1 Ara	2 Ara	no subst.	1 Ara	2 Ara
E. xiangfangensis	RBG-18-0107-1	I	+	+	6	94,2	5,8	0,0	43,1	55,1	1,8
E. xiangfangensis	RBL-17-0437-1	I	+	+	8	99,6	0,4	0,0	32,6	59,9	7,5
E. xiangfangensis	RPF-17-0260-1	I	+	+	6	99,8	0,2	0,0	34,5	61,4	4,1
E. xiangfangensis	BK4615	I	-	+	16	99,9	0,1	0,0	36,3	56,2	7,6
E. xiangfangensis	RBK-17-0394-1	I	-	+	16	96,7	3,3	0,0	30,1	62,4	7,5
E. xiangfangensis	RBG-17-0018-1	I	-	-	4	100,0	0,0	0,0	100,0	0,0	0,0
E. xiangfangensis	RPT-16-0254-2	II	-	-	4	100,0	0,0	0,0	100,0	0,0	0,0
E. xiangfangensis	RBG-18-0378-1	III	-	-	4	100,0	0,0	0,0	100,0	0,0	0,0
E. xiangfangensis	RBF-18-0224-1	III	+	-	4	100,0	0,0	0,0	100,0	0,0	0,0
E. xiangfangensis	RBL-17-0298-2	IV	-	-	4	100,0	0,0	0,0	100,0	0,0	0,0

b

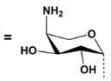


$LA_{hexa/hepta}$ + 0 Ara4N:
R_1, R_2 = H

$LA_{hexa/hepta}$ + 1 Ara4N:
R_1= H, R_2 = Ara4N or R_1= Ara4N, R_2 = H

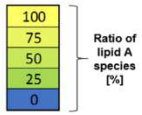
$LA_{hexa/hepta}$ + 2 Ara4N:
R_1, R_2 = Ara4N

Ara4N =



c

Species	Isolate	*arn* operon	*phoPQ*	Ara4N-substitution of lipid A species		
				no colistin		
				no subst.	1 Ara	2 Ara
E. roggenkampii	RPB-17-0516-2	+	+	83,9	16,1	0,0
E. ludwigii	F8789	+	+	93,9	6,1	0,0
E. bugandensis	RBG-17-0246-1	+	+	58,1	40,1	1,8
E. cloacae	RPK-18-0157-1	+	+	70,6	28,0	1,4
E. chengduensis	RBK-18-0141-1	+	+	62,5	36,1	1,4
E. cancerogenus	RPK-18-0479-1	+	+	62,1	34,1	3,8
E. mori	RBL-17-0354-2	+	+	94,3	5,7	0,0
E. kobei	BK6664	+	+	93,5	6,5	0,0
E asburiae	BK5433	+	+	76,9	22,7	0,4
E. vonholyi	F-147	+	truncated	96,8	3,2	0,0
E. wuhouensis	F00728	-	+	100,0	0,0	0,0



d

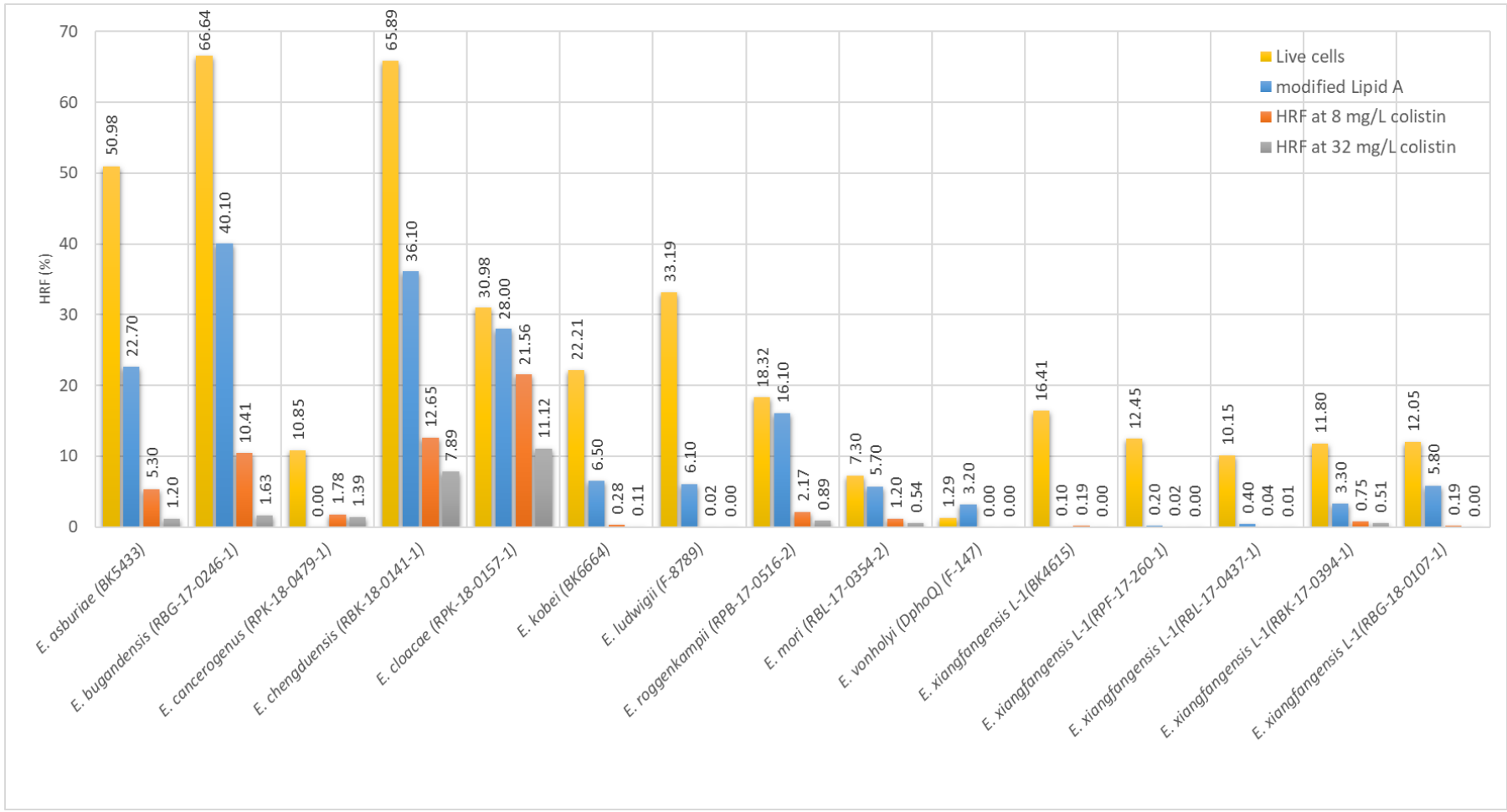

HRF (%)
Live cells
modified Lipid A
HRF at 8 mg/L colistin
HRF at 32 mg/L colistin
E. asburiae (BK5433)
E. bugandensis (RBG-17-0246-1)
E. cancerogenus (RPK-18-0479-1)
E. chengduensis (RBK-18-0141-1)
E. cloacae (RPK-18-0157-1)
E. kobei (BK6664)
E. ludwigii (F-8789)
E. roggenkampii (RPB-17-0516-2)
E. mori (RBL-17-0354-2)
E. vonholyi (DphoQ) (F-147)
E. xiangfangensis L-1(BK4615)
E. xiangfangensis L-1(RPF-17-260-1)
E. xiangfangensis L-1(RBL-17-0437-1)
E. xiangfangensis L-1(RBK-17-0394-1)
E. xiangfangensis L-1(RBG-18-0107-1)

Figure 3: Genetic determinants of heteroresistance in *Enterobacter* species. Comparative genomics of colistin-resistant and -sensitive isolates revealed that *arnBCADTEF* is necessary for colistin heteroresistance. In *Enterobacterales*, *arnBCADTEF* expression is controlled by the TCS PhoPQ, while the activity of the PhoPQ pathway is inhibited by the small transmembrane protein MgrB. Deletion mutants were created in *E. xiangfangensis* L-1 isolate RBK-17-0394-1 (WT) and studied for the MIC and HRF (number of cells recovered on LB agar plate containing 8 or 32 mg/L of colistin / number of cells inoculated). MIC was calculated by BMD while HRF was calculated by PAP test. Analysis of mutants showed the PhoPQ/arnBCADTEF axis is absolutely required for the colistin heteroresistance, while MgrB acts as a negative regulator of *arnBCDATEF* resulting in colistin susceptibility. As reported recently by Kang et al.[58] for *E. cloacae*, PmrAB did not contribute to colistin resistance in *E. xiangfangensis*.

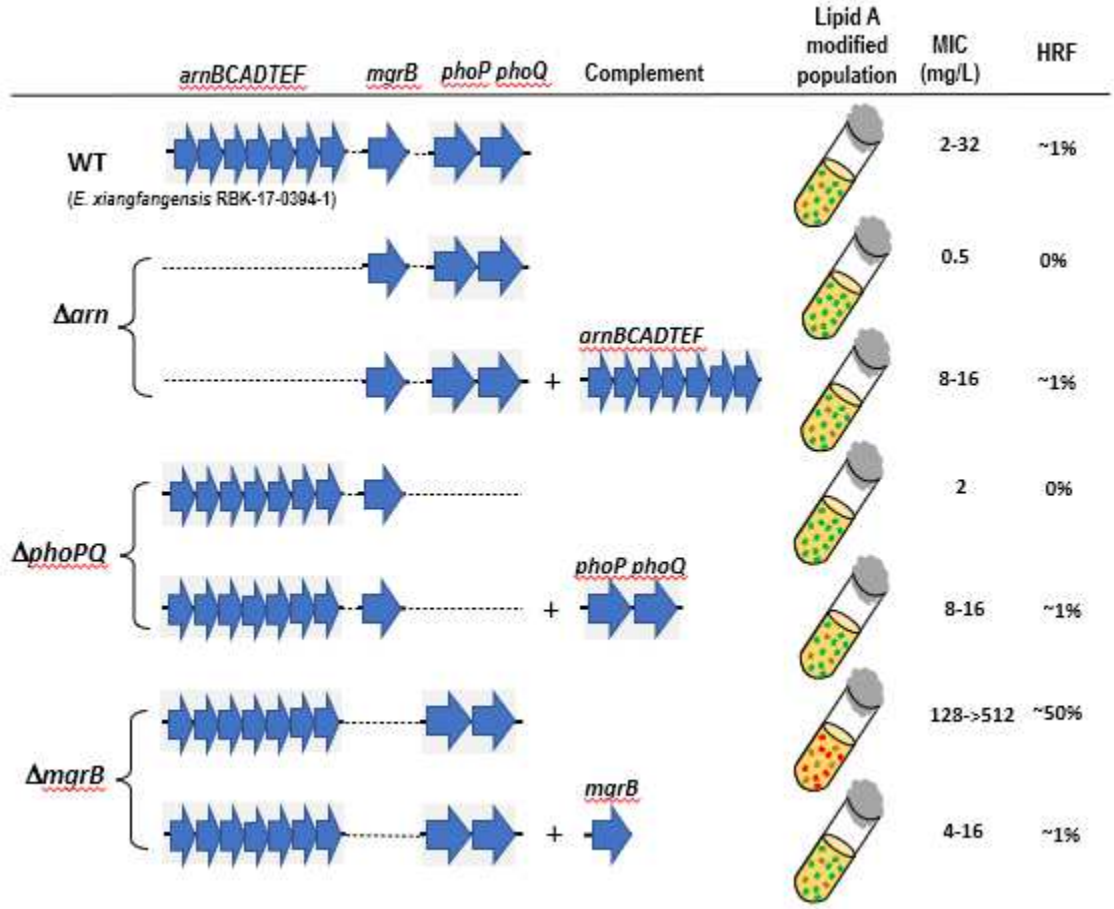


Figure 4: Heteroresistance capabilities of *phoP-phoQ* hybrid and *mgrB* complemented mutants. The hybrid complement mutants were created by combining *phoP* and *phoQ* genes either of *E. xiangfangensis* RBK-17-0394 (Ex), *E. bugandensis* RBG-17-0246-1 (Eb), and *E. roggenkampii* RPB-17-0516-2 (Er). Plasmids harbouring *phoP-phoQ* hybrids were transferred to Exf394Δ*phoPQ*. Complementation of the *phoQ* from the isolates exhibiting higher colistin heteroresistance i.e. Eb and Er to Δ*phoPQ* mutant of *E. xiangfangensis* RBK-17-0394-1 resulted in the colistin resistance equivalent to respective parent strains. Complementation of the Δ*mgrB* mutant of *E. xiangfangensis* RBK-17-0394-1 with the *mgrB* from Eb (which has identical amino acid sequence as of Er) resulted in slightly lesser inhibition due to changes in the binding energy (detailed in Supplementary Figure 7).

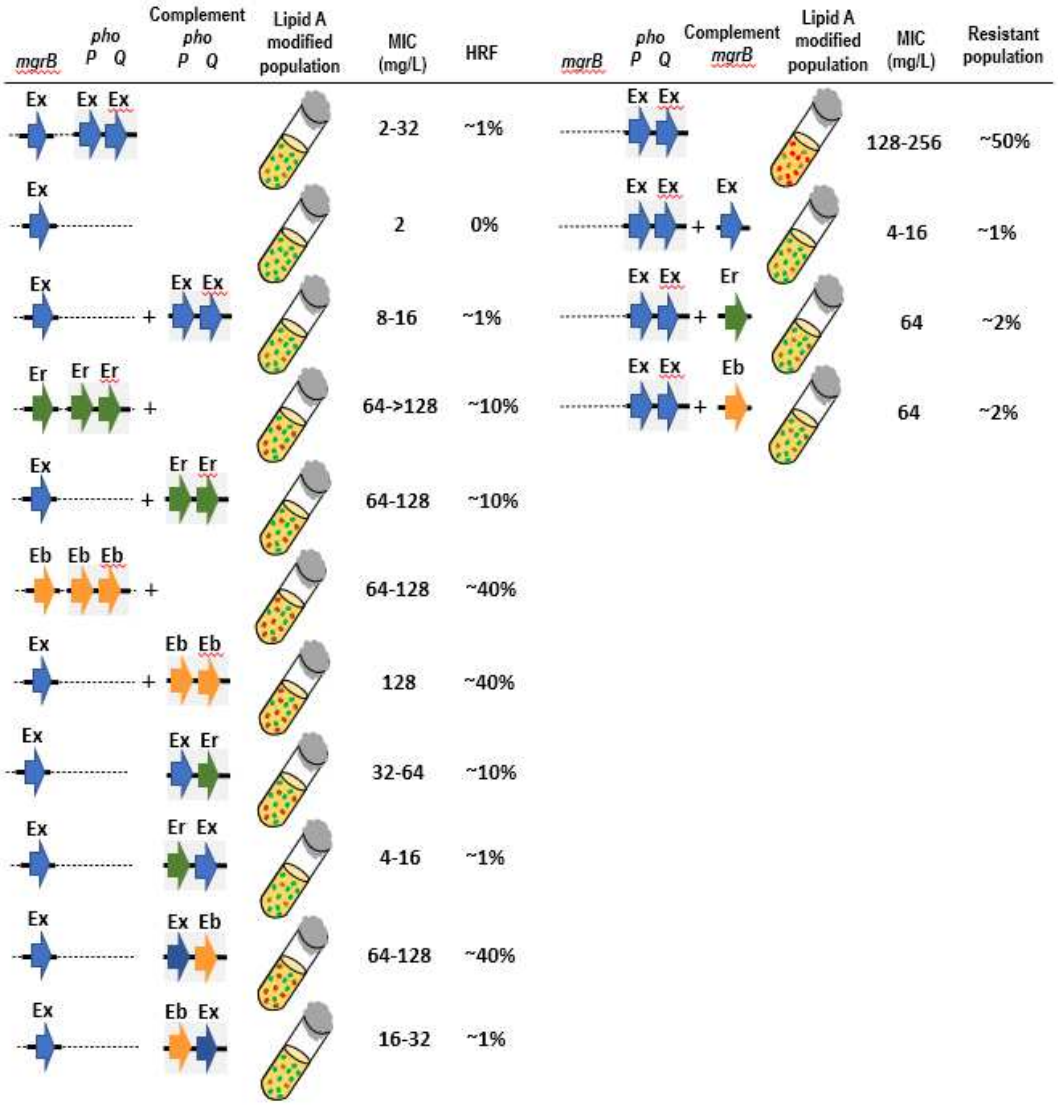
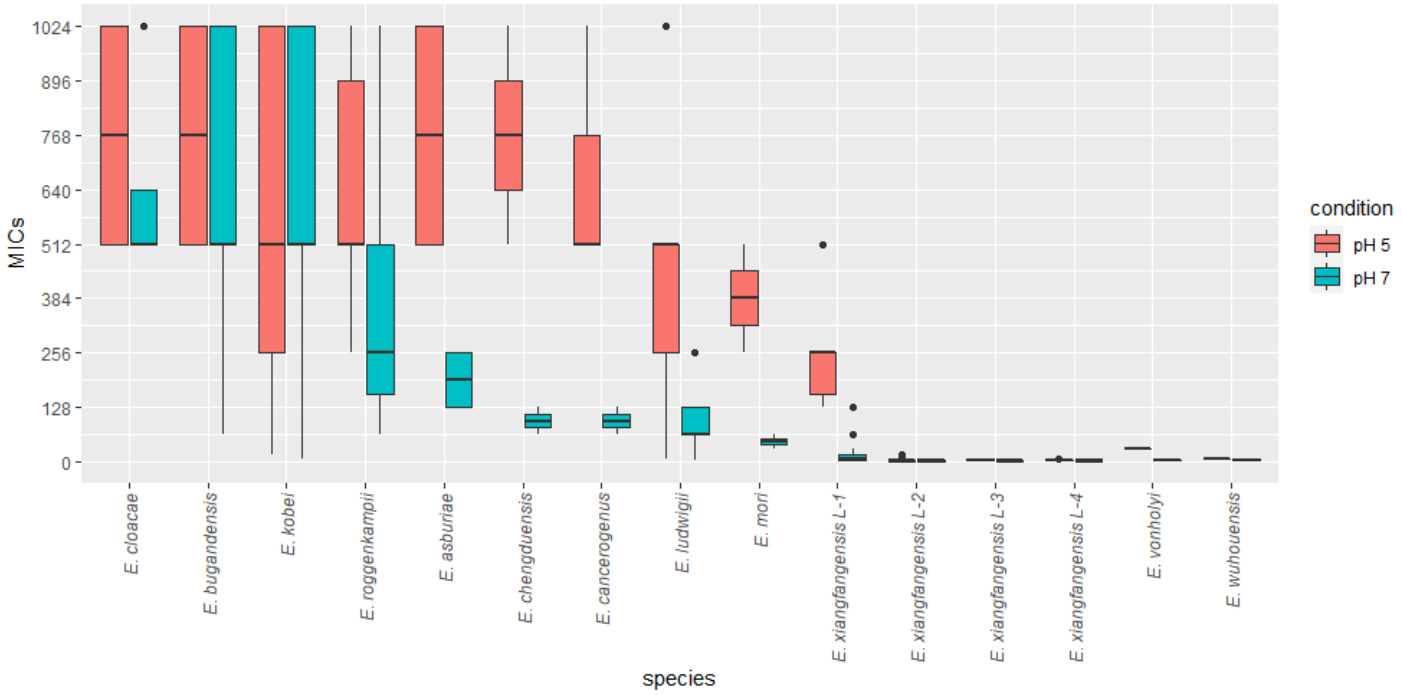


Figure 5: Minimum inhibitory concentration (MIC) (a) and heteroresistance frequency (HRF) (b) of different *Enterobacter* species. The MIC and HRF were studied by BMD assay and PAP test for all the 165 isolates of 12 species observed in this study (Supplementary data 1). To depict the species-wise range of MICs and HRFs, the lowest and highest, average-low and average-high MIC and HRF of each of the species were calculated. The lowest and highest MICs/HRFs of individual species are shown by line, while box represents average low and average high MICs/HRFs. The red boxes depict the elevated MICs at pH 5. The MICs/HRFs for *E. xiangfangensis*, *E. wuhouensis*, and *E. vonholyi* are shown in the inset. The MICs and HRFs for each individual isolate are provided in Supplementary data 1.

(a)



951 (b)

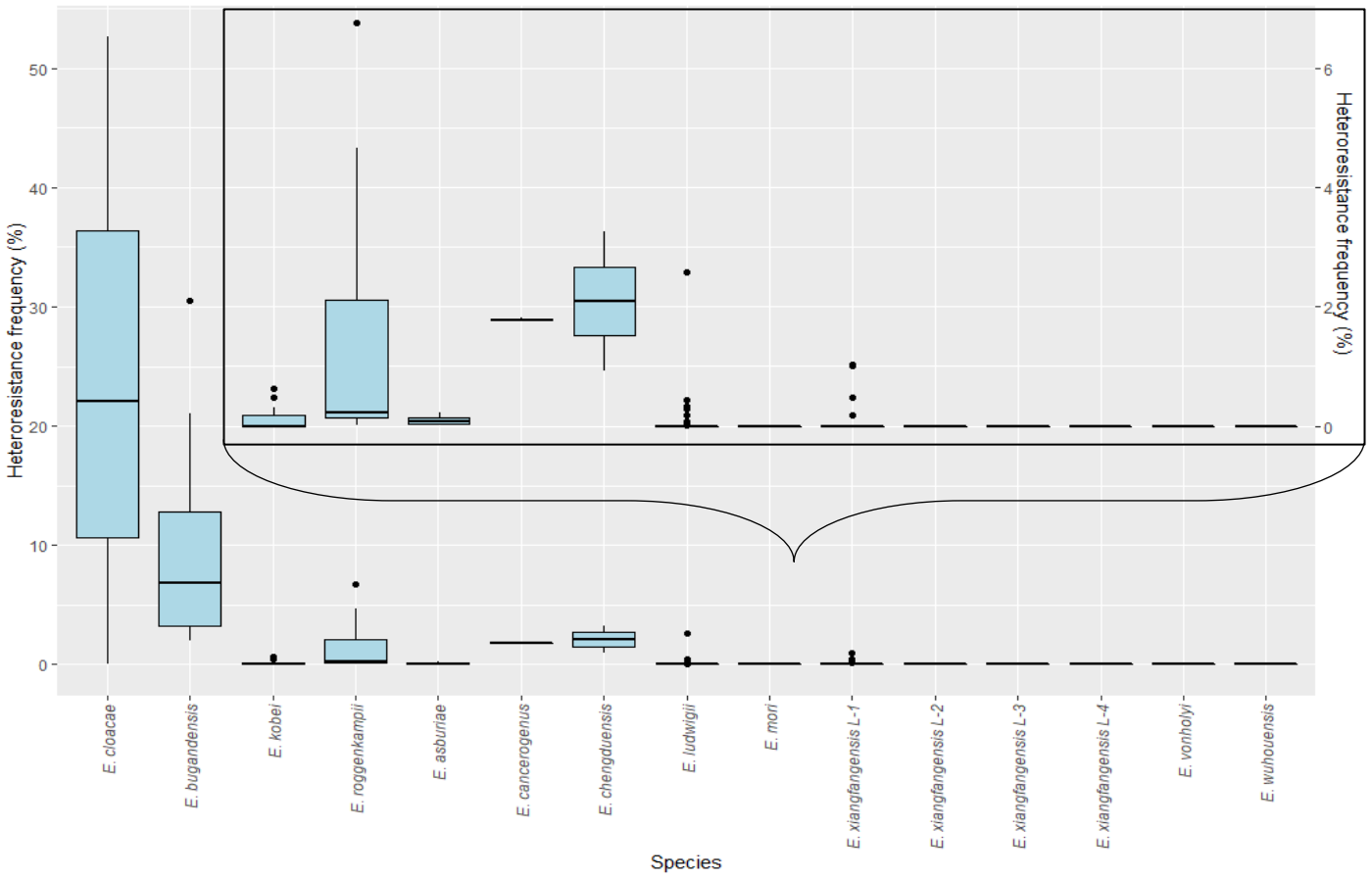

Heteroresistance frequency (%)
50
40
30
20
10
0
6
4
2
0
Heteroresistance frequency (%)
E. cloacae
E. bugandensis
E. kobei
E. roggenkampii
E. asburiae
E. cancerogenus
E. chengduensis
E. ludwigii
E. mori
E. xiangfangensis L-1
E. xiangfangensis L-2
E. xiangfangensis L-3
E. xiangfangensis L-4
E. vonholyi
E. wuhouensis
Species

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Figure 6: Model for mechanistic details of colistin heteroresistance. The PhoQ component senses environmental conditions (for e.g., low pH and cationic antimicrobial peptide (CAMP)), which results in a change in its conformation leading to phosphorylation of PhoP. Phosphorylated PhoP binds to the promoter of many genes, including *arnBCDATEF*. Expression of *arnBCDATEF* results in the addition of L-Ara4N to lipid A, which changes the overall charge of the outer membrane causing colistin resistance. Under normal growth conditions (LB, pH 7) PhoQ of the overall growing population is strongly inhibited by mgrB[57]. **(a)** As a result of microenvironmental changes experienced during growth, a portion of the isogenic growing population has activated PhoQ with concomitant expression of the *arnBCDATEF* loci that result in L-Ara4N-modified lipid A. Only these cells survive when exposed to colistin (HRF+). Overall, levels of modified lipid A are low. **(b)** In the presence of subinhibitory concentrations of the cationic antimicrobial peptide such as colistin (2 mg/L), the antibiotic penetrates the outer membrane and is sensed by PhoQ weakening interactions with MgrB and thereby releasing expression of *arnBCDATEF*. This results in L-Ara4N-modified lipid A in a higher number of bacteria. When exposed to colistin, these populations show relatively high survival levels (HRF++). **(c)** As a result of low pH (pH 5) during growth conditions, MgrB is prevented from an interacting with PhoQ, resulting in heightened expression of the *arnBCDATEF* cassette and subsequent L-Ara4N-modified lipid A in higher number of cells. Quantitative lipid A analysis confirms these observations and shows that these populations exhibit increased survival (HRF+++). **(d)** As a result of low pH (pH 5) and antimicrobial peptide colistin during growth, PhoQ experiences a cumulative effect, and thus MgrB inhibition is strongly prevented, resulting in heightened expression of *arnBCDATEF* cassette and subsequent arabinose modified lipid A in a higher number of cells. Quantitative analysis of lipid A confirms these observations, and these populations exhibit increased survival (HRF++++). (Core sugars and fatty acids of the LPS are only drawn symbolically and do not reflect exact structures)

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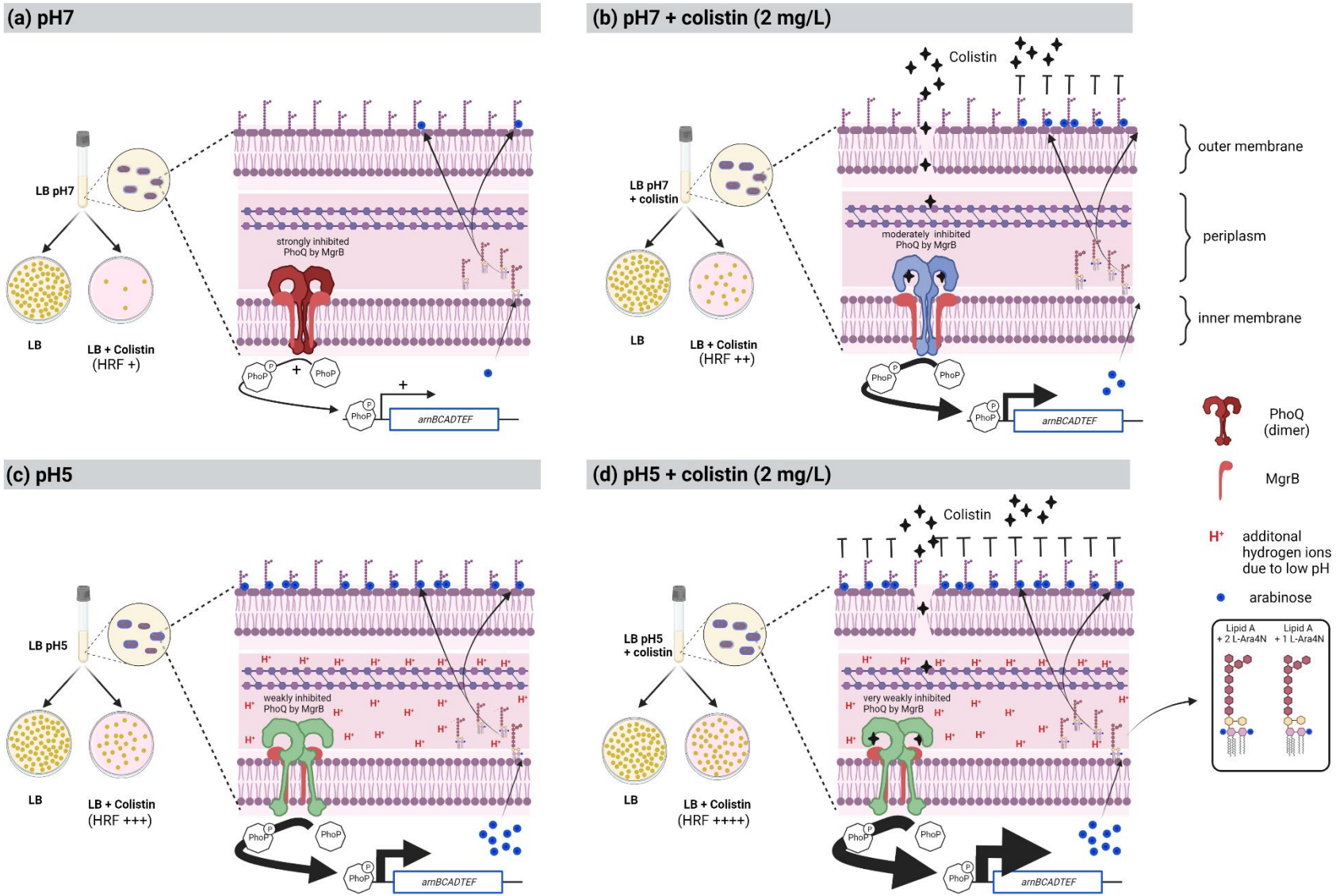
(a) pH7
LB pH7
LB
LB + Colistin
(HRF +)
strongly inhibited
PhoQ by MgrB
PhoP
arnBCADTEF
(b) pH7 + colistin (2 mg/L)
Colistin
LB pH7
+ colistin
LB
LB + Colistin
(HRF ++)
moderately inhibited
PhoQ by MgrB
outer membrane
periplasm
inner membrane
PhoQ
(dimer)
MgrB
H+ additonal
hydrogen ions
due to low pH
arabinose
Lipid A
+ 2 L-Ara4N
Lipid A
+ 1 L-Ara4N
(c) pH5
LB pH5
LB
LB + Colistin
(HRF +++)
weakly inhibited
PhoQ by MgrB
(d) pH5 + colistin (2 mg/L)
Colistin
LB pH5
+ colistin
LB
LB + Colistin
(HRF ++++)
very weakly inhibited
PhoQ by MgrB

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Supplementary Files

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- Supplementarydata6phoPphoQarnmgrBmutations.xlsx
- Supplementarydata7SNVs.xlsx