Research Square

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Persistent phoQ upregulation and sigma-factor modulation link polymyxin resistance to broader stress responses in Klebsiella pneumoniae ST11

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Abstract

Background

Polymyxin resistance in *Klebsiella pneumoniae* frequently involves lipid A remodeling controlled by the PhoPQ and PmrAB two-component systems. However, interactions between these pathways and sigma factor-mediated stress responses remain incompletely understood in high-risk lineages.

Objective

To determine how polymyxin B exposure affects targeted transcriptional responses in the polymyxin-resistant *K. pneumoniae* sequence type 11 (ST11) clinical isolate Pol16 and to relate these responses to resistance- and stress-associated genomic alterations.

Methods

Pol16 was characterized by broth microdilution, whole-genome sequencing, and targeted sequence analysis. Expression of *phoQ, pmrD, pmrB, rpoD, rpoS*, and *rpoE* was quantified by reverse transcription quantitative PCR in exponential-phase cultures grown with or without 4 µg/mL polymyxin B. Expression was normalized to rpoC and calculated relative to *K. pneumoniae* ATCC 700603.

Results

Pol16 belonged to ST11 and carried a large deletion affecting *mgrB*, together with alterations in genes associated with two-component systems, lipid A modification, and σE regulation. phoQ was upregulated under both growth conditions and increased further following polymyxin exposure. Exposure also induced marked increases in *rpoD* (5.291-fold) and *rpoS* (2.453-fold). *pmrD* remained upregulated but did not differ significantly between the conditions tested, whereas *pmrB* remained below the calibration baseline. Although *rpoE* remained below baseline, its expression increased significantly after exposure.

Conclusion

The integrated genomic and transcriptional profile supports persistent PhoPQ signaling, potentially favored by *mgrB* disruption, accompanied by RpoD/RpoS-associated stress responses. The limited *pmrB* response and distinct *rpoE* behavior highlight a regulatory configuration that broadens the understanding of polymyxin resistance in a clinically important ST11 lineage.

Introduction

The prevalence of polymyxin resistance has increased among clinically important Gram-negative pathogens, including *K. pneumoniae*, a frequent cause of community- and hospital-acquired infections. Multidrug-resistant *K. pneumoniae* is recognized as a critical public-health threat, underscoring the need to clarify resistance mechanisms and improve control strategies [1–5].

Polymyxins are used as last-line agents against infections caused by multidrug-resistant Gram-negative bacteria. Their cationic residues bind to negatively charged phosphate groups in lipid A, disrupting the bacterial envelope. Resistance commonly arises when the net negative charge of lipid A is reduced through the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (pEtN) [6–11].

In *K. pneumoniae*, lipid A remodeling is regulated mainly by the PhoPQ, PmrAB, and CrrAB two-component systems. PhoPQ can activate the *arnBCADTEF-ugd locus* directly and can also communicate with PmrAB through the connector PmrD. Loss of the negative regulator MgrB is a frequent route to enhanced PhoPQ signaling and polymyxin resistance [5, 12]. In parallel, sigma factors coordinate basal transcription and stress-responsive programs, but changes in sigma-factor transcripts cannot by themselves establish sigma-factor activity or a direct contribution to resistance [13, 14].

This study characterized the polymyxin-resistant *K. pneumoniae* ST11 clinical isolate Pol16 and examined the expression of *phoQ*, *pmrD*, and *pmrB* genes, together with the sigma-factor genes *rpoD* (σ70), *rpoS* (σS), and *rpoE* (σE), during growth with and without polymyxin B. Sequence alterations in polymyxin resistance-associated genes and in regulators of the σE pathway were also assessed.

Materials and Methods

Bacterial isolate and antimicrobial susceptibility testing

The *K. pneumoniae* isolate Pol16 was recovered from a urinary tract infection in a hospitalized patient in northeastern Brazil. Species identification was performed using the Vitek 2 system (bioMérieux, Marcy-l'Étoile, France). The polymyxin B minimum inhibitory concentration was determined by broth microdilution using the Policimbac kit (Probac, Brazil). The antimicrobial susceptibility profile is provided in Supplementary Table S1 (Online Resource 1).

Whole-genome sequencing and in silico analysis

Genomic DNA was extracted with the PureLink Genomic DNA Mini Kit (Invitrogen) and sequenced on the Illumina NextSeq 550 platform (Illumina, San Diego, CA, USA) using 2 × 75-bp paired-end reads. Reads were assembled with Velvet and the VelvetOptimiser script, using N50 optimization for k-mer selection [15]. Coding sequences were predicted and annotated with the NCBI Prokaryotic Genome Annotation Pipeline v6.1 [16]. The whole-genome shotgun sequence is available under GenBank accession JAJQQO000000000.1.

Resistance determinants were investigated with BIGSdb v1.34.0 [17], ResFinder v4.1 [18], and the Comprehensive Antibiotic Resistance Database [19]. Virulence-associated *loci* were assessed with BIGSdb, VirulenceFinder v2.0 [20-22], and VFDB Analyzer [23,24]. Kleborate v2.2.0 was used for species confirmation, multilocus sequence typing, and characterization of ICEKp-associated *loci*, capsule, K *locus*, and O antigen. Plasmid replicons were identified with PlasmidFinder v2.1 [21,22], and the sequence type was verified in PubMLST [25]. Tools were run with default settings unless otherwise stated; software details are provided in Supplementary Table S3 (Online Resource 1).

Coding and predicted protein sequences of *pmrA*, *pmrB*, *phoP*, *phoQ*, *crrA*, *crrB*, *arnA*, *arnB*, *arnC*, *arnD*, *arnT*, *mgrB*, *pmrD*, *ugd*, *eptA*, *eptB*, *rseA*, *rseB*, *rseP*, *surA*, *degS*, *rsd*, *rpoD*, *rpoS*, and *rpoE* were compared in Geneious Prime

v2023.0.4 and SnapGene 2023. *K. pneumoniae* ATCC 700603 (GenBank accession NZ_AOGO00000000.1) was used as the sequence reference. Alterations are summarized in Supplementary Table S2 and Figure S2 (Online Resource 1).

Primer design, RNA extraction, and RT-qPCR

Primers were designed from conserved target-gene regions using PrimerQuest. Specificity, GC content, melting temperature, and potential dimer formation were evaluated within the silico PCR and OligoAnalyzer. Amplification efficiency was determined from standard-curve slopes, and melt curves were inspected for single products and absence of detectable primer-dimer or nonspecific amplification (Table 1; Supplementary Figure S1, Online Resource 1). The *rpoC* primer pair was obtained from Gomes et al. [26].

Pol16 and *K. pneumoniae* ATCC 700603 cultures were grown in cation-adjusted Mueller-Hinton broth at 37 °C with shaking. Pol16 was cultivated with or without 4 µg/mL polymyxin B and harvested during exponential growth at OD600 = 0.5. Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific), treated with RQ1 RNase-Free DNase (Promega), and quantified with a NanoDrop Lite spectrophotometer (Thermo Scientific).

Complementary DNA was synthesized with the EasyScript Reverse Transcriptase/EasyScript cDNA Synthesis Kit (Applied Biological Materials). Expression of *phoQ*, *pmrD*, *pmrB*, *rpoD*, *rpoS*, and *rpoE* genes was quantified on an Amplio96 instrument (Loccus) with SYBR Green I detection and 25 ng of total RNA equivalent per reaction. *rpoC* was used as the endogenous control, and polymyxin-susceptible *K. pneumoniae* ATCC 700603 as the calibration strain. Three independent biological replicates were analyzed, each in technical triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method.

Statistical analysis

Between-condition comparisons were performed using Student's *t* test with Bonferroni correction for normally distributed data and the Wilcoxon test for non-normally distributed data. A two-sided *P* value < 0.05 was considered statistically significant. Analyses were performed in R using the stats package [27].

Ethics approval

The study was approved by the Research Ethics Committee of the University of Pernambuco, Brazil (reference 265.604).

Table 1 Primers used for RT-qPCR and amplification-performance parameters

Gene	Product/function	Primer sequence, 5'-3'	Amplicon bp	Tm, °C	GC, %	Efficiency, %	Slope
phoQ	PhoPQ sensor histidine kinase	F ACCCCTTTGCGCTGATAGAC R TGAGCCGGGAATTACACCC	83	64.2 64.2	55.0 55.0	98.537	−3.358
pmrD	PhoPQ-PmrAB connector protein	F GGCGACAGGCTTCAATCTCT R TACAGGACAACGCTTCGGC	87	64.1 64.3	55.0 57.9	97.980	−3.371
pmrB	PmrAB sensor histidine kinase	F TCACTCCTATCGTACTCAGC R TCGAGGGTCAGATTGAGG	94	60.1 60.0	50.0 55.6	102.936	−3.254
rpoD	RNA polymerase sigma factor RpoD (σ70)	F CCGAGCTGTCTCAGGAAGAG R GTTGTCGTCATCGCTGTTGT	83	63.3 63.0	60.0 50.0	92.097	−3.527
rpoS	RNA polymerase sigma factor RpoS (σS)	F CGCGAGTTGTCCCATAAG R CGTTCAGACGCAGCATAC	103	59.8 60.4	55.6 55.6	95.941	−3.423
rpoE	RNA polymerase sigma factor RpoE (σE)	F CGGATCAGGTCCTTGTTG R CGCGACCTTATGCTGATAG	86	60.0 60.0	55.6 52.6	96.980	−3.396
rpoC	RNA polymerase β' subunit	F TATTCTGGTTCCACGCAACA R GGATACAACGGAACGCACTT	97	62.0 62.6	45.0 50.0	96.048	−3.420

Tm, melting temperature; GC, guanine-cytosine content. Target-gene primers were designed in this study; the *rpoC* primer pair was reported by Gomes et al. [26].

Results

Genomic findings associated with polymyxin resistance

Pol16 was classified as polymyxin resistant and assigned to sequence type ST11. Targeted sequence comparisons identified missense substitutions and deletions in genes encoding two-component systems and their regulators, including *pmrB*, *crrB*, *pmrD*, and *mgrB* genes. A large deletion affected *mgrB* gene, the negative regulator of PhoQ. Alterations were also detected in the σE regulatory genes *rseB*, *rseP*, and *degS* and in genes involved in L-Ara4N synthesis or transfer (*arnA*, *arnC*, *arnD*, and *arnT*). The remaining genes examined were intact or contained only synonymous substitutions relative to *K. pneumoniae* ATCC 700603. Detailed alterations and alignments are provided in Supplementary Table S2 and Figure S2 (Online Resource 1).

Target-gene expression during polymyxin B exposure

In Pol16 grown without polymyxin B, *phoQ* and *pmrD* expression was 2.542-fold and 3.648-fold, respectively, relative to the ATCC 700603 calibration strain. Expression of the *pmrB*, *rpoD*, *rpoS*, and *rpoE* genes remained below

the calibration baseline under this condition (Fig. 1).

Exposure to polymyxin B was associated with a significant increase in *phoQ* expression compared with untreated Pol16, while *pmrD* remained at a similar level at the different conditions. *pmrB* expression remained below the calibration baseline. The most pronounced change at the different conditions tested was observed for *rpoD*, which reached 5.291-fold expression relative to the calibration strain during polymyxin exposure. *rpoS* increased to 2.453-fold. Although *rpoE* increased significantly between Pol16 distinct culture conditions, its expression remained below the ATCC 700603 calibration baseline (Fig. 1).

Discussion

The findings of this study indicate that polymyxin resistance in *K. pneumoniae* Pol16 is associated with a regulatory profile dominated by persistent *phoQ* upregulation and accompanied by changes in transcriptional responses related to bacterial stress adaptation. The large deletion affecting *mgrB*, together with increased *phoQ* expression in both untreated and polymyxin-exposed conditions, provides a coherent genomic and transcriptional context for enhanced PhoPQ-associated signaling. Moreover, polymyxin exposure further increased *phoQ* expression and was accompanied by increased transcription of *rpoD* and *rpoS*, suggesting that exposure to the antibiotic intensifies not only the response linked to polymyxin resistance but also broader mechanisms involved in cellular adaptation to stress.

The PhoPQ system is one of the major regulatory pathways involved in polymyxin resistance in *K. pneumoniae*, particularly through modifications of lipid A that reduce the interaction of polymyxins with the bacterial outer membrane [12, 36, 37]. Inactivation of *mgrB*, a negative regulator of PhoPQ, is a well-established mechanism associated with constitutive activation of this system and polymyxin resistance [38, 39, 43, 44]. In Pol16, the presence of a large *mgrB* deletion together with constitutively increased *phoQ* transcription strongly supports the involvement of this regulatory axis in the resistant phenotype. The additional increase in *phoQ* expression during polymyxin exposure suggests that, despite an already elevated basal transcriptional state, the system remains responsive to antibiotic-induced envelope stress. This observation is relevant because PhoPQ activity is influenced by multiple environmental and regulatory signals and may be further modulated under conditions that perturb membrane homeostasis [40–42].

The transcriptional behavior of *pmrD* and *pmrB* provides additional insights into the organization of this response. The *pmrD* gene was upregulated in Pol16 relative to the calibration strain under both conditions, although its expression did not increase significantly following polymyxin exposure. PmrD functions as a connector between PhoPQ and PmrAB by stabilizing phosphorylated PmrA, thereby contributing to the regulatory network controlling lipid A remodeling [38, 40, 45, 46]. Its sustained expression in Pol16 may therefore reflect a regulatory background already adapted to polymyxin resistance rather than an acute response specifically induced by antibiotic exposure. In contrast, *pmrB* transcript levels remained below those observed in ATCC 700603 in both conditions. Taken together, these findings suggest that the transcriptional response detected in Pol16 is more strongly associated with the PhoPQ axis than with increased *pmrB* expression. This pattern is also compatible with evidence that PhoPQ can promote expression of lipid A modification pathways in *K. pneumoniae* without requiring strong transcriptional activation of the PmrAB system [38, 39].

Beyond these classical polymyxin resistance pathways, exposure of Pol16 to polymyxin B was associated with marked changes in sigma-factor transcription. *rpoD* showed a 5.291-fold increase during polymyxin exposure. RpoD (σ70) is the primary housekeeping sigma factor and controls a broad range of genes required for bacterial

growth and cellular maintenance [14, 28]. Its increased expression may therefore reflect a substantial transcriptional adjustment to the physiological disturbance caused by polymyxin B rather than a resistance-specific response. At the same time, *rpoS*, encoding the general stress sigma factor σS, was also induced during exposure. RpoS coordinates bacterial adaptation to multiple adverse conditions and contributes to survival during environmental and physiological stress [31, 32]. The simultaneous increase in *rpoD* and *rpoS* suggests that polymyxin exposure triggers a broader transcriptional reorganization in Pol16, combining maintenance of essential cellular functions with activation of stress-adaptation pathways.

The σE pathway adds another layer to this response because it is closely associated with envelope homeostasis in Gram-negative bacteria. Pol16 carried sequence alterations in *degS*, *rseB*, and *rseP*, including a 171-bp deletion in *degS* and a 21-bp deletion together with an amino-acid substitution in *rseB*. These genes participate in the regulatory cascade controlling σE release through the RseA anti-sigma factor [29, 30]. Although *rpoE* transcription remained below the calibration strain baseline, its expression increased significantly after polymyxin exposure. Thus, the σE-associated response appears to remain responsive to antibiotic-induced envelope stress despite the genomic alterations identified in components of its regulatory pathway. The combination of genomic variation and inducible *rpoE* expression suggests a potentially modified regulatory architecture that may influence how Pol16 responds to membrane damage [29, 30, 33–35].

Collectively, these findings support a model in which polymyxin resistance in Pol16 is associated with a constitutively altered regulatory background centered on the *mgrB*–PhoPQ axis, while polymyxin exposure further amplifies *phoQ* transcription and recruits broader stress-related responses involving RpoD, RpoS, and, to a lesser extent, RpoE. Rather than representing isolated transcriptional events, these changes indicatet coordinated adaptation to the membrane and physiological stress imposed by polymyxin B. The comparatively stable expression of *pmrD* and low *pmrB* transcription further indicate that the different regulatory components involved in polymyxin resistance do not respond uniformly to antibiotic exposure.

Because the study focused on a single clinical isolate and a targeted set of transcripts, these findings primarily define the regulatory profile of Pol16 and provide a basis for further mechanistic investigation. Functional analyses of pathway activity and lipid A composition, as well as evaluation of additional polymyxin-resistant isolates, will be important to determine how broadly this transcriptional pattern is present. Nevertheless, the combined genomic and transcriptional data provide evidence that the polymyxin response in Pol16 extends beyond classical resistance determinants and involves a broader regulatory adaptation to antibiotic-induced stress.

Conclusion

Together, these findings support a resistance-associated regulatory profile predominantly linked to the *mgrB*–PhoPQ axis and accompanied by a broader transcriptional response to polymyxin-induced stress. This integrated genomic and transcriptional perspective expands the understanding of regulatory responses associated with polymyxin resistance in high-risk ST11 *K. pneumoniae*.

Declarations

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Competing interests

The authors declare no financial or non-financial interests that are directly or indirectly related to this work.

Author contributions

Michelly Maria Pereira e Oliveira: Methodology, Investigation, Writing - original draft. Paula Mariana Salgueiro de Souza: Writing - review and editing. Patrícia Francisca Gama da Silva: Investigation. Lara Valesca Mendonça da Costa Santos: Formal analysis. Rodrigo Tenório Gomes Pereira: Investigation. Márcia Maria Camargo de Morais: Conceptualization, Writing - review and editing. Anna Carolina Soares Almeida: Conceptualization, Supervision, Data curation, Writing - review and editing.

Data availability

The whole-genome shotgun sequence of *K. pneumoniae* Pol16 is available in GenBank under accession JAJQQO000000000.1. Additional data supporting the study are included in the article and its supplementary information.

Ethics approval

This study was approved by the Research Ethics Committee of the University of Pernambuco, Brazil (reference 265.604).

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Figures

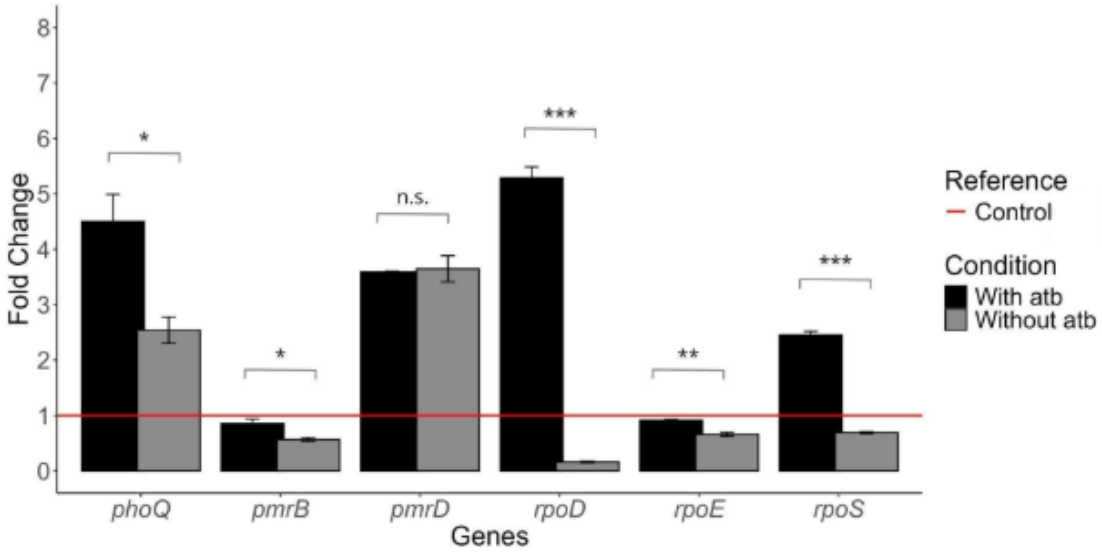


Figure 1

Relative expression of *phoQ*, *pmrB*, *pmrD*, *rpoD*, *rpoE*, and *rpoS* in *K. pneumoniae* Pol16 grown with 4 µg/mL polymyxin B (black bars) or without antibiotic (gray bars). Expression was normalized to *rpoC* and calculated relative to *K. pneumoniae* ATCC 700603 using the $2^{-\Delta\Delta Cq}$ method. The red line denotes the calibration baseline (fold change = 1). Bars show mean ± standard deviation for three biological replicates. Brackets compare the two Pol16 growth conditions by Student's *t* test. **P* < 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; n.s., not significant

Supplementary Files

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