

Comparative phenotypic and proteomic analysis of colistin-exposed *Pseudomonas aeruginosa*

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Abstract

Introduction The emergence of colistin resistance threatens the treatment of *Pseudomonas aeruginosa* infections.

Methods In this study, in vitro development of colistin resistance was investigated using comparative phenotypic and proteomic analysis of *P. aeruginosa* ATCC 9027, its 14-day colistin sub-MIC exposed strain (Col-E1), and 10-day antibiotic-free cultured Col-E1 strain (Col-E2). Antibiotic susceptibility, morphology, virulence factors, and proteomic changes were assessed using disc-diffusion, agar-based, spectrophotometry, SEM, and iTRAQ-LC-MS/MS methods.

Results Colistin-exposed strains decreased susceptibility to colistin while remaining susceptible to other antibiotics. Col-E1 reduced the cell lengths by 17.67% and the colony size by 36.16% compared to the initial strain. The reduction remained in Col-E2. The pyocyanin production was reduced in Col-E1 ($p=0.025$, Tukey HSD) and increased again in Col-E2 ($p=0.005$, Tukey HSD). In contrast, no significant changes in elastase, protease, rhamnolipid, pyoverdine, and biofilm production were observed ($p>0.05$, Tukey HSD). In Col-E1, the proteome analysis showed 135 differentially expressed proteins (DEPs) of which 94 DEPs (69.23%) maintained their expression change in Col-E2. Among DEPs, 82 were involved in metabolism and protein synthesis. Some DEPs (6/135) played a role in stress response such as GrpE (fold change: 14.93) and Hmp (fold change: 12.08). In particular, membrane proteins like OprD, DdlB, and OprI showed significant colistin response with fold change of -8.47, 6.43 and 6.19, respectively.

Conclusions In summary, colistin response in *P. aeruginosa* seemed to affect morphology, production of pyocyanin, and proteins of metabolism, protein synthesis, stress response and membrane.

Keywords Colistin, morphology, proteome, *Pseudomonas aeruginosa*, virulence factors.

Introduction

Pseudomonas aeruginosa is an important nosocomial pathogen causing a wide range of infections, particularly for immunocompromised patients. For the last decade, it emerges as a big threat to public health due to the ability to resist

to multiple antibiotics.¹ Under exposure to sub-minimum inhibitory concentration (MIC) values of antibiotics, the bacterium could significantly increase its MIC values over time.² Several studies showed that sub-MIC values induced the alteration of bacterial characteristics and

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virulence expression but detailed mechanisms are not known.³

Colistin (polymyxin E) is commonly used in human and animal health care.⁴ By interacting with lipid A of lipopolysaccharide (LPS), colistin causes damage to the outer membrane of Gram-negative bacteria resulting in cell death.⁵ Resistance to polymyxins involves outer membrane change, upregulation of efflux systems, and acquisition of some resistance determinants. Mutations in *lpxA*, *lpxC*, and *lpxD* lead to dysfunction of lipid A biosynthesis, resulting in loss of LPS; overexpression of outer membrane protein OprH prevents the binding of colistin to the LPS, and can also result in colistin resistance.⁶

Proteomics is a powerful tool for studying molecular responses to antibiotics.⁷ However, limited proteomic studies have been performed to understand the colistin resistance mechanisms.⁸ In this work, the colistin adaptive resistance of *P. aeruginosa* was investigated under the perspectives of morphology, virulence factors, and proteome.

Methods

Bacterial strains

P. aeruginosa ATCC 9027 (American Type Culture Collection, USA) was the original strain in this study. The microdilution assay in Mueller Hinton (MH) broth following a previous study⁹ was used to assess colistin MIC values of *P. aeruginosa* ATCC 9027 and generate Col-E1

strain. We collected the bacteria at the well next to the MIC well, where the growth of *P. aeruginosa* was still evident for the subsequent exposure. Antibiotic concentrations used were adjusted based on the obtained MIC values. The exposure process was performed for 14 days to achieve the 14-day colistin sub-MIC exposed strain (Col-E1). Col-E1 was then continuously exposed to colistin-free medium for 10 days. MIC values of colistin were checked daily using the microdilution method mentioned previously.⁹

Antibiotic susceptibility testing

P. aeruginosa ATCC 9027, Col-E1, and Col-E2 strains were cultured in MH broth (HiMedia, India) for 24 h at 37°C. Then the diluted cultures ($OD_{600nm} = 0.08$) were spread on MH agar. The commercial antibiotic paper discs of different antibiotics (meropenem (Me) (10 µg), tobramycin (Tb) (10 µg), ceftazidime (Cz) (30 µg), gentamicin (Ge) (10 µg), ciprofloxacin (Ci) (5 µg), cefepime (Cm) (30 µg), piperacillin/ tazobactam (Pt) (100/10 µg), imipenem (Im) (10 µg)) (NAM KHOA Biotek Company, Vietnam) were placed on the surface of the agar and incubated overnight at 37°C. The inhibition zones were measured and evaluated following Clinical and Laboratory Standards Institute (CLSI) 2022 and CLSI 2023 (USA).

Morphology and virulence factor assessment

Colony morphology and cell length observation

P. aeruginosa ATCC 9027, Col-E1, and Col-E2 strains were cultured in tryptic soy broth (TSB) for 24 h at 37°C. The cultures were adjusted to $OD_{600nm} = 0.08$, then 10^6 dilution cultures were prepared to ensure that the colony count on each Petri dish remained below 15. This practice aimed to facilitate optimal morphology observation when spreading the cultures on tryptic soy agar as the recommendation from a previous study.¹⁰ Following the spreading, the dishes were incubated overnight at 37°C. Colony morphology was observed using a stereo microscope. The experiment was performed in triplicate.

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Bacterial cell length was measured by using scanning electron microscopy (SEM). The sample preparation for SEM was performed following our previous study.¹¹ Each strain was embedded on plastic discs by culturing overnight in LB broth. The plastic discs were subsequently washed with 1X PBS buffer and fixed by 10% formalin for 24 hours. Following fixation, specimens were rinsed with 1X PBS and dehydrated by series of graded ethanol (25%, 50%, 70%, 80%, 90%, 100%). For air drying, the specimens were treated with t-butyl alcohol for 24 hours at 4°C. Finally, the individual cell length was analyzed by SEM COXEM machine (South Korea) and ImageJ software (NIH, USA). Ten cells from each sample were measured.

Virulence factor assessment

Elastase, protease, rhamnolipid, pyocyanin, pyoverdine, and biofilm production of *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2 were analyzed. *P. aeruginosa* ATCC 9027 was used as a positive control for the tests because it could produce tested virulence factors in this study. For negative control, *Escherichia coli* ATCC 4157 was used for protease, pyocyanin and pyoverdine tests, while *Staphylococcus aureus* ATCC 29213 was used for elastase test.¹² Each virulence test was performed in triplicate.

Elastase, protease, and rhamnolipid testing: The elastase, protease, and rhamnolipid production of *P. aeruginosa* strains were observed by spotting method on elastin–nutrient plate agar (0.3% elastin), skim milk agar, methylene blue (MB) and cetyltrimethylammonium bromide (CTAB) containing agar (HiMedia Laboratories, USA and Sigma-Aldrich, USA), respectively. Then the plates were incubated at 37°C for 24 h and at 30°C 48 h for elastase test. The enzymatic activity was visualized by the halos around the spot cultures.

For pyocyanin testing, the strains were cultured in glycerol-alanine (Gly-Ala) broth medium to maximize the yield of pyocyanin production.¹³ After culturing overnight at 37°C with shaking, the culture was centrifuged at 6000 rpm for 20 min to collect the supernatant. After filtering, 3 mL of the supernatant were mixed well with 1.8 mL of chloroform (VN-CHEMSOL

Co., Ltd., Vietnam). The raw pyocyanin (red layer) was extracted by mixing the blue layer with 0.2 N HCl (2:1). The pyocyanin absorbance was measured at 520 nm using BioTek Synergy HTX Multimode Reader (Agilent, USA). The pyocyanin concentration (µg/mL) was estimated by multiplying OD_{520nm} by 17.072 which is the molar extinction coefficient of pyocyanin at 520 nm.¹⁴

For pyoverdine testing, King B broth (HiMedia Laboratories, USA) was used to maximize the yield of pyoverdine production. After culturing overnight at 37°C with shaking, the culture was centrifuged at 6000 rpm for 20 min to collect the supernatant. After filtering, the fluorescence intensity of the supernatant was measured by BioTek Synergy HTX Multimode Reader at an excitation 405 nm and emission 450 nm.¹⁵

Biofilm testing: The crystal violet (HiMedia Laboratories, USA) staining method was used to test for biofilm production.¹⁶ The OD_{550nm} was measured using BioTek Synergy HTX Multimode Reader.

iTRAQ LC-MS/MS proteomic analysis

Quantitative iTRAQ- LC-MS/MS analysis

Total protein extraction was performed following the procedure described in a previous study.¹⁷ Proteins were trypsinized and labeled with iTRAQ tags from the Reagent 8-plex kit (Sigma-Aldrich, USA). iTRAQ-labeled peptide mixtures were analyzed using strong-cation-exchange (SCX) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Ultimate 3000 LC system and 5800 MALDI-TOF/TOF analyzer were used to run the samples. Protein identification and quantification using the MS/MS spectra was performed using Protein Pilot Software 4.5 (Applied Biosystems) according to the Swiss-Prot/UniProt database.¹⁸

Quantitative reverse transcription PCR (RT-qPCR) analysis

Total RNA was extracted using Monarch® Total RNA Miniprep Kit (New England Biolabs, USA). The quantity and quality of the extracted RNA were assessed using BioTek Synergy HTX Multimode Reader. Then, it was transcribed into complementary DNA (cDNA) using the

SensiFAST™ cDNA Synthesis Kit and qPCR was performed using SensiFAST™ SYBR No-ROX Kit (Meridian Bioscience, USA). The thermal cycle setting included 2 min at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The *rpoD* and *rplU* were used as internal controls to normalize fold changes.^{19,20} Primers in this study were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) – Table S1. The fold change was evaluated against *P. aeruginosa* ATCC 9027 according to the $2^{-\Delta\Delta C_t}$ method from a study of Kumar and Lorand 2021.²¹ Values greater than 1 were up-regulated genes, while values smaller than 1 were down-regulated genes. The experiment was triplicated.

Data analysis

IBM® SPSS® Statistics 20.0 (IBM Corp., USA) was used to analyze the data. The Tukey HSD test was used to determine the difference in morphology and virulence factor production of *P. aeruginosa* strains. The p-value was set to be <0.05. Raw MS/MS data were analyzed by using ProteinPilot Software 4.5 (AB SCIEX). The proteins were considered to be differentially expressed if their iTRAQ ratios were ≥ 2 (up-regulation) and ≤ 0.5 (or ≤ 2) (down-regulation). For protein identification, a threshold applied was >0.05 (CI, 10%) with setting ProtScore at 2.0 and the false discovery rate (FDR) <1%. The differentially expressed proteins (DEPs) were analyzed by the Gene Ontology (GO) annotation from the UniProt database. A protein-protein interaction network was constructed using the web resource STRING 11.5. To classify the protein functions, UniProt, STRING 11.5, and PANTHER 17.0 were applied.

Results

Antibiotic susceptibility profile of *P. aeruginosa* under colistin exposure

After continuously being exposed to sub-MIC of colistin for 14 days, the colistin MIC value of *P. aeruginosa* ATCC 9027 increased from 4 µg/mL to 16 µg/mL (Figure S1). And after 10 days of being cultured back in antibiotic-free medium, the colistin MIC dropped to 8 µg/mL. Furthermore, Col-E1 and Col-E2 did not develop

cross resistance to other antibiotics, including meropenem, tobramycin, ceftazidime, gentamicin, ciprofloxacin, cefepime, piperacillin/tazobactam and imipenem (Table S2).

Morphology of *P. aeruginosa* under colistin exposure

The colony sizes of Col-E1 and Col-E2 were significantly different from the *P. aeruginosa* ATCC 9027. The average colony sizes of Col-E1 and Col-E2 were smaller than the original strain, which were 3.92 ± 0.51 mm, 3.10 ± 0.25 mm, and 6.14 ± 0.19 mm, respectively ($p < 0.05$, Tukey HSD) – Figure 1.

The cell length of *P. aeruginosa* ATCC 9027 (2.49 ± 0.45 µm) decreased significantly after exposure to colistin in which Col-E1 was 2.05 ± 0.21 µm and Col-E2 was 1.90 ± 0.23 µm ($p < 0.05$, Tukey HSD) – Figure 2.

Elastase, protease, rhamnolipid, pyocyanin, pyoverdine, and biofilm production of *P. aeruginosa* under colistin exposure

The obtained data indicated that *P. aeruginosa* ATCC 9027 reduced the production of pyocyanin under the exposure to colistin ($p = 0.025$, Tukey HSD test) and increased again after colistin was removed from the culture medium ($p = 0.005$, Tukey HSD test). However, the production of elastase, protease, rhamnolipid, pyoverdine, and biofilm remained in both Col-E1 and Col-E2 ($p > 0.05$, Tukey HSD test) – Table 1.

Proteome response of *P. aeruginosa* to colistin

The same set of 947 different proteins were identified in *P. aeruginosa* ATCC 9027, Col-E1 and Col-E2 strain using iTRAQ LC-MS/MS. DEPs were determined when quantitatively comparing Col-E1 and Col-E2 strain to the initial strain *P. aeruginosa* ATCC 9027. In total, for both Col-E1 and Col-E2, more up-regulated proteins (Col-E1: 108, Col-E2: 101) than down-regulated proteins (Col-E1: 27, Col-E2: 41) were found (cut-off: ≥ 2 : up-regulation or ≤ 2 : down-regulation). The fold change ranged from -8.47 to 17.95. Interestingly, 94 DEPs in Col-E1 kept their expression change even after 10 days being cultured in antibiotic-free medium (Col-E2),

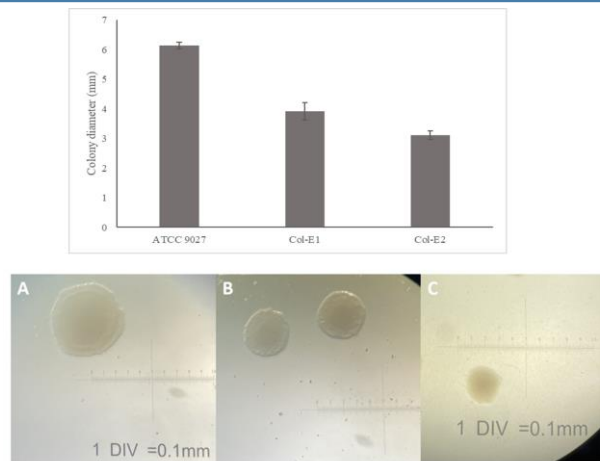


Figure 1. Colony morphology of *P. aeruginosa* in response to colistin exposure. Upper image: average colony size from three replications of each strain: *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2. Lower image: representative colony image of (A) *P. aeruginosa* ATCC 9027, (B) Col-E1, and (C) Col-E2. The bacterium was cultured on TSA at 37°C for 24h.

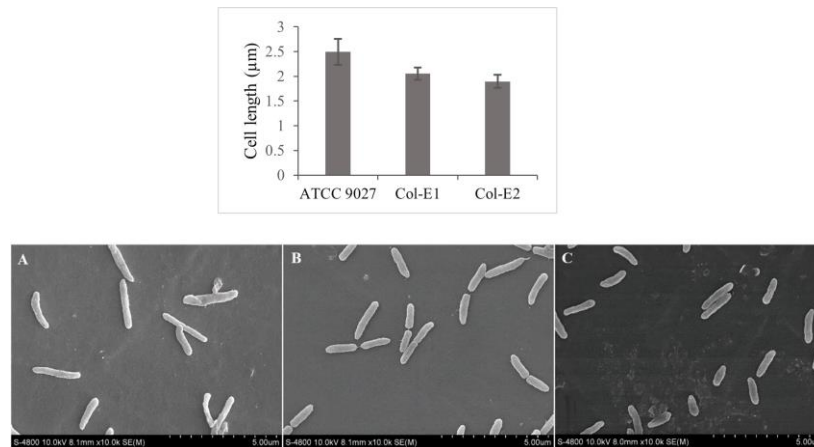


Figure 2. Cell morphology of *P. aeruginosa* in response to colistin exposure. Upper image: Average cell size of 10 cells of each strain: *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2. Lower image: representative scanning electron microscopy (SEM) images of (A) *P. aeruginosa* ATCC 9027, (B) Col-E1, and (C) Col-E2.

Table 1. Elastase, protease, rhamnolipid, pyocyanin, pyoverdine, and biofilm production of *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2.

Virulence factors	<i>P. aeruginosa</i> ATCC 9027	Col-E1	Col-E2
Elastase (mm)	3.58 ± 0.63	3.50 ± 0.50	3.67 ± 1.61
Protease (mm)	3.17 ± 0.14	2.75 ± 0.25	2.66 ± 0.38
Rhamnolipid (mm)	2.92 ± 0.76	2.44 ± 0.64	2.58 ± 0.40
Pyocyanin (μg/mL)*	1.26 ± 0.13	0.40 ± 0.24	1.60 ± 0.42
Pyoverdine (excitation: 405 nm, emission: 450 nm)	175.46 ± 19.97	169.92 ± 6.37	210.39 ± 3.71
Biofilm (OD _{550nm})	5.95 ± 1.34	2.42 ± 2.00	7.96 ± 3.85

There were no significant differences in the production of elastase, protease, rhamnolipid, pyoverdine, and biofilm between these strains ($p > 0.05$, Tukey HSD). The pyocyanin production of Col-E1 was reduced significantly ($*p = 0.025$, Tukey HSD), while Col-E2 increased the pyocyanin production again ($*p = 0.005$, Tukey HSD).

including 82 up- and 12 down-regulated proteins (Figure S2, Table S3). On the other hand, 40 DEPs reverted their expression to the initial state before the colistin exposure, and only 1 protein turned from up to down regulation after colistin was withdrawn (PA0038; fold change: Col-E1: 2.74, Col-E2: -3.09) – Table S4.

The DEPs related to metabolism and protein synthesis

To classify the function of DEPs of *P. aeruginosa* in responding to colistin, PANTHER 17.0 was used. Two big classified functional groups were molecular function and biological process (Figure 3). Most DEPs belonged to structural molecule activity, catalytic activity in molecular function group (Figure 3A) and metabolic process, and cellular process in biological process group (Figure 3B). From STRING analysis, there were 22 clusters detected from DEPs of Col-E1. The biggest cluster included 25 ribosomal proteins (Figure S3). They were all up-regulated and the fold change ranged from 2.63 to 10.05.

The DEPs related to stress response

There were 7 up-regulated proteins (GrpE, Hmp, MsrA, LptF, PhoP, PA2575, and PA4061) and 1 down-regulated protein (TrxB1) related to stress response detected in *P. aeruginosa* under colistin exposure as classified by protein function from UniProt. The fold change ranged from -2.68 to 14.93 (Figure 4, Tables S3, S4).

The DEPs related to membrane modification

Following UniProt, 16 DEPs with membrane-related functions were detected, including 8 up-regulated and 8 down-regulated proteins (fold change: -8.47 to 2.10). The up-regulated membrane proteins were DdlB (6.43), OprI (6.19), SltB1 (3.68), Ffh (3.63), BamD (2.91), PagL (2.64), WapR (2.37), and PA5378 (2.17). The down-regulated proteins were OprD (-8.47), PA2760 (-2.47), PilP (-2.36), LptE (-2.34), BamE (-2.33), Alr (-2.16), PA1288 (-2.15), and PA0833 (-2.05) – Figure 4, Table S3, S4.

Quantitative reverse transcription PCR (RT-qPCR) analysis

Selected DEPs found via proteomic analysis also presented the same trend of change at gene expression level. However, the fold change from the RT-qPCR analysis was overall lower than that from the iTRAQ LC-MS/MS analysis. It was 1.26, 1.30, 2.00, 1.05 and 0.57 for *grpE*, *hmp*, *fur*, *mvaT* and *oprD*, respectively (Figure S4, Table S5).

Discussion

After exposure to the sub-MIC of colistin for a while, *P. aeruginosa* ATCC 9027 increased MIC value of colistin, which was consistent with previous observations that the pathogen could become less susceptible to an antibiotic to which it was exposed² and changed its properties.³ Interestingly, the increasing fold in MIC for colistin is relatively low, only 2-4 folds compared to the increasing fold induced by other antibiotics.²² Furthermore, long-term exposure to colistin luckily did not trigger cross resistance to other antibiotics.

As colistin inhibited cell growth by disrupting cell membrane, it is under expectation that colony size was reduced under colistin exposure.²³ Besides, shorter cell length observed after colistin exposure was also consistent with other studies about *P. aeruginosa* morphological changes in responding to colistin exposure in vitro or during treatment.^{24,25} It has been known that cell length or cell size was dependent to the growth rate which positively links to DNA content and protein synthesis,^{26,27} thus it is understandable that antibiotics which interfere with cellular process have impact on cell size.

Prolonged exposure to sub-MIC levels of colistin in *P. aeruginosa* did not significantly impact the production of most virulence factors, except for pyocyanin. Pyocyanin production was observed to decrease in the presence of colistin, returning to normal upon colistin removal. It has been known that the sub-MIC of antibiotics, including colistin, could inhibit the production of pyocyanin.²⁸ In a previous study, elastase exceptionally increased activity when being incubated with colistin.²⁹ Unlike colistin, ciprofloxacin exposure significantly reduced

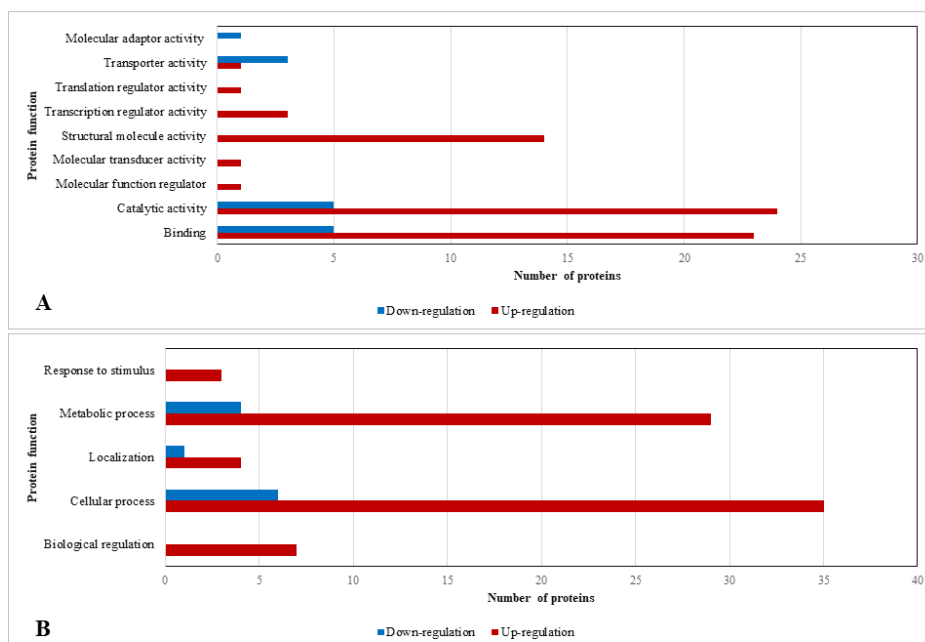


Figure 3. The function classification of DEPs of *P. aeruginosa* after exposure to colistin (Col-E1). (A) Molecular function and (B) Biological process were grouped by PANTHER 17.0 (<http://www.pantherdb.org/>).

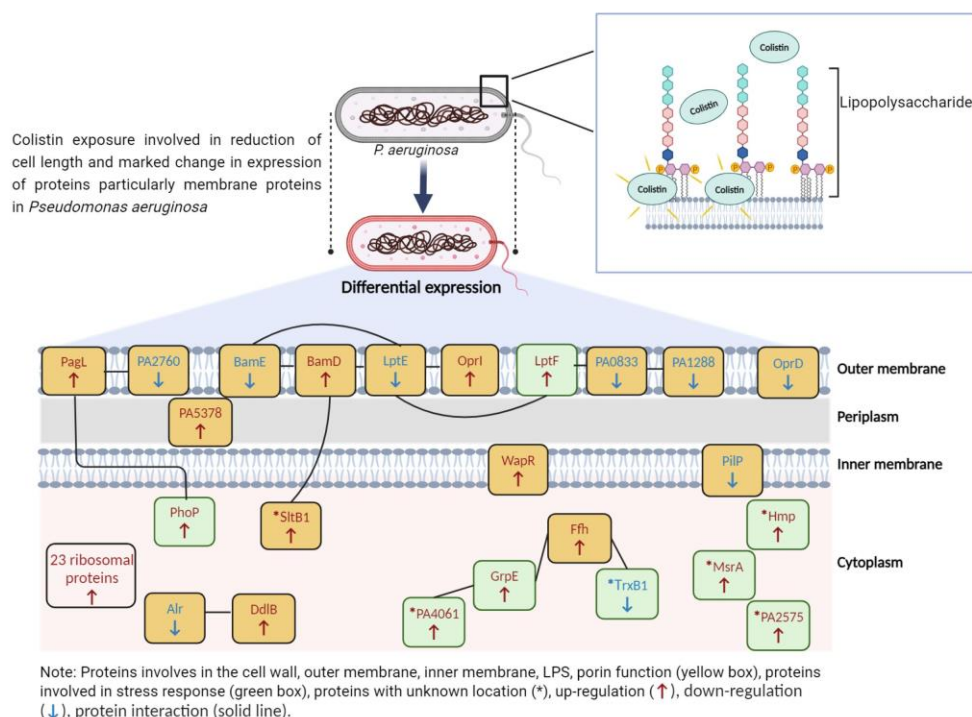


Figure 4. Proteomic response of *Pseudomonas aeruginosa* to colistin exposure. The colistin responsive proteins primarily involved membrane, stress response and protein synthesis. OprI and OprD alternative expression was shown to be particularly important in colistin response. The protein function, location, and interactions were classified by STRING 11.5 (<https://string-db.org/>) and UniProt (<https://www.uniprot.org/>).

elastase, protease, and biofilm production in *P. aeruginosa*.²² In colistin-resistant derivatives of *Acinetobacter baumannii* ATCC 19606, reduction of biofilm production and loss of LPS were also noted.³⁰

At protein level, virulence-related proteins did not show much change in responding to colistin. For instance, Fur (3.53) and BfrB (2.08), which were responsible for iron uptake of *P. aeruginosa*,³¹ were slightly up-regulated in our study. Regarding biofilm production, MvaT (2.37), which took part in biofilm formation,³² was also only slightly up-regulated. LpxA (1.15), LpxC (1.01), and LpxD (-1.53) which were shown to be involved in the virulence of colistin-resistant pathogens, especially in reducing biofilm production,³⁰ also did not change expression under colistin exposure. PtsP (-1.20), which contributed to the pyocyanin production,³³ showed a minor decrease in expression in the presence of colistin.

Most found DEPs were related to metabolism and protein synthesis. Among ribosomal proteins, RplJ had the highest fold change (10.05). The highest upregulated protein involved in metabolism was MetF (14.32). This protein indirectly influenced protein synthesis via the methionine biosynthesis process.³⁴ The lowest down protein of the metabolism group was PyrC (-3.15), which is involved in pyrimidine biosynthesis. The pyrimidine biosynthesis process is known to be crucial for bacterial proliferation,³⁵ thus downregulation of PyrC is an appropriate adaptation to antibiotics stress including colistin.

For proteins involved in stress response, GrpE (Col-E1: 14.93, Col-E2: 11.22) and Hmp (Col-E1: 12.08, Col-E2: 19.31) had the highest fold change. GrpE prevented the aggregation of stress-denatured proteins when *P. aeruginosa* faced to hyperosmotic or heat shock,³⁶ while Hmp was essential in response to nitrosative stress or NO₂ pollutant condition.³⁷ LptF (Col-E1: 3.39, Col-E2: 3.21) was an OmpA-like outer membrane protein, reported to be involved in resistance to oxidative stress response of *P. aeruginosa*.³⁸ PhoP (Col-E1: 2.56 and Col-E2: 2.41) was a member of the two-component regulatory system

PhoP/PhoQ which played a role in the resistant regulation to cationic antibiotics.³⁹

Among membrane protein, OprI was highly up-regulated (Col-E1: 6.19 and Col-E2: 6.82). It may be because OprI compensated the decline of OprD for the integrity of the outer membrane.⁴⁰ Other membrane DEPs including LptE and BamD, BamD and BamE, Alr and DdlB, particularly PagL and PhoP, were shown to be involved in disrupting polymyxin penetration.⁴¹ Some DEPs such as PA2760, PA0833, PA1288, and PA5378, could be potential factors in the colistin adaption of *P. aeruginosa* which would need further investigation. The summary of DEPs and their cellular locations indicated clearly the involvement of membrane proteins and protein-synthesis related proteins in the colistin response of the bacterium (Figure 4).

In general, even when colistin was removed from the culture for many generations during 10 days of continuous culture, a great number of DEPs maintained their expression trend while only a few, such as PA4061 (2.17 to 1.54), Trb1 (-2.68 to -1.50) and OprD (-8.47 to -1.94), returned to their normal expression. OprD was thought to be the most prevalent mechanism for carbapenem resistance⁴² and colistin response⁴³ in *P. aeruginosa*. Previous studies showed that the resistance phenotype could be reverted, a characteristic feature of adaptive resistance, when the antibiotic stress is removed.⁴⁴ However, in our study, most of the changed phenotypic characteristics and many DEPs remained after the exposure to colistin was stopped. We assumed that colistin-exposed *P. aeruginosa* may have epigenetic memory⁴⁵ or changes have been made in its genome. It would be more likely the epigenetic changes as the expected resistance-related mutation rates were lower than the observed survival rates in sub-MIC of antibiotics.⁴⁶

Sub-MIC exposure can clearly cause long-term effect on the protein expression changes of colistin-exposed *P. aeruginosa*, thus the use of antibiotics must be done with even greater caution. It is obvious that surviving *P. aeruginosa* under sub-MIC colistin exposure develop sustainable colistin resistance without acquiring

any external resistant genes, thus antibiotic dosage and duration must be reasonable to eradicate the pathogen completely. In addition, combination therapy could be one option. An in vitro study showed that combining colistin with aminoglycoside, carbapenems, fluoroquinolones, and others could significantly improve outcomes for infections caused by multidrug-resistant *Klebsiella pneumoniae*, a common antibiotic-resistant Gram-negative bacterium like *P. aeruginosa*.⁴⁷ However, some in vivo studies on combination of colistin and carbapenem, although displaying a positive effect, did not provide significant improvement.⁴⁸⁻⁵⁰ One good point from the studies is that the toxicity or adverse effect of colistin combination was similar to monotherapy.⁴⁸⁻⁵⁰

Moreover, DEPs identified in our study could potentially serve as biomarkers for colistin-resistant isolates. Rapid detection of resistance is essential for clinicians to administer more effective treatment strategies as well as to prevent the spread of resistant isolates.

This study provides valuable insights into the response of *P. aeruginosa* ATCC 9027 to colistin exposure and identifies potential biomarkers for colistin resistance. However, it has some significant limitations. First, it did not provide direct evidences showing the role of each protein to the colistin resistance development or phenotypic changes. Second, the changes in genome, transcriptome or metabolome of the bacterium in responding to colistin were not analyzed. Third, whether this response is the same for different strains and different antibiotics is still not investigated. Therefore, further investigations should be performed to unlock this complicated problem.

Conclusions

After colistin exposure, surviving *P. aeruginosa* decreased colony size, cell length, and the production of pyocyanin, but did not dramatically change production of elastase, protease, rhamnolipid, pyoverdine, and biofilm. Luckily, surviving *P. aeruginosa* did not develop high-fold increased resistance to colistin and cross resistance to other antibiotic classes. A substantial

proportion of DEPs maintained the expression changes even though *P. aeruginosa* was no longer in colistin containing environment, suggesting an epigenetic regulation memory in responding to antibiotics.

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Conflicts of interest: All authors – none to declare.

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Data availability: All data generated or analyzed during this study are included in this published article.

References

1. Yero D, Díaz-Lobo M, Costenaro L, et al. The *Pseudomonas aeruginosa* substrate-binding protein Ttg2D functions as a general glycerophospholipid transporter across the periplasm. *Commun Biol*. 2021;4:448. <https://doi.org/10.1038/s42003-021-01968-8>
2. Sandoval-Motta S, Aldana M. Adaptive resistance to antibiotics in bacteria: a systems biology perspective. *WIREs Syst Biol Med*. 2016;8:253-67. <https://doi.org/10.1002/wsbm.1335>
3. de Andrade JPL, de Macêdo Farias L, Ferreira JFG, et al. Sub-inhibitory concentration of piperacillin-tazobactam may be related to virulence properties of filamentous *Escherichia coli*. *Curr Microbiol*. 2016;72:19-28. <https://doi.org/10.1007/s00284-015-0912-9>

4. Gogry FA, Siddiqui MT, Sultan I, Haq QMohdR. Current update on intrinsic and acquired colistin resistance mechanisms in bacteria. *Front Med (Lausanne)*. 2021;8:677720. <https://doi.org/10.3389/fmed.2021.677720>
5. Döbelmann B, Willmann M, Steglich M, et al. Rapid and consistent evolution of colistin resistance in extensively drug-resistant *Pseudomonas aeruginosa* during morbidostat culture. *Antimicrob Agents Chemother*. 2017;61:e00043-17. <https://doi.org/10.1128/AAC.00043-17>
6. Saleh NM, Hesham MS, Amin MA, Samir Mohamed R. Acquisition of colistin resistance links cell membrane thickness alteration with a point mutation in the *lpxD* gene in *Acinetobacter baumannii*. *Antibiotics (Basel)*. 2020;9:164. <https://doi.org/10.3390/antibiotics9040164>
7. Tsakou F, Jersie-Christensen R, Jenssen H, Mojsoska B. The role of proteomics in bacterial response to antibiotics. *Pharmaceuticals (Basel)*. 2020;13:214. <https://doi.org/10.3390/ph13090214>
8. Hashemi MM, Holden BS, Coburn J, et al. Proteomic analysis of resistance of Gram-negative bacteria to chlorhexidine and impacts on susceptibility to colistin, antimicrobial peptides, and ceragenins. *Front Microbiol*. 2019;10:210. <https://doi.org/10.3389/fmicb.2019.00210>
9. Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. *Pathogens*. 2021;10(2):165. <https://doi.org/10.3390/pathogens10020165>
10. Sousa AM, Machado I, Nicolau A, Pereira MO. Improvements on colony morphology identification towards bacterial profiling. *J Microbiol Methods*. 2013;95:327-35. <https://doi.org/10.1016/j.mimet.2013.09.020>
11. Nguyen NHB, Pham TTV, Huynh TQ, Nguyen TH, Nguyen TTH. Sample preparative procedure for *Pseudomonas aeruginosa* observation under scanning electron microscope. *Vietnam J Biotechnol*. 2022;20:717-26. <https://doi.org/10.15625/1811-4989/16934>
12. Garcia D de O, Timenetsky J, Martinez MB, Francisco W, Sinto SI, Yanaguita RM. Proteases (caseinase and elastase), hemolysins, adhesion and susceptibility to antimicrobials of *Stenotrophomonas maltophilia* isolates obtained from clinical specimens. *Braz J Microbiol*. 2002;33:157-62. <https://doi.org/10.1590/S1517-83822002000200012>
13. Ahamed F, Devnath P. Extraction, purification and characterization of pyocyanin produced by *Pseudomonas aeruginosa* and evaluation for its antimicrobial activity. *Int Res J Biol Sci*. 2017;6:1-7.
14. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol*. 1990;172:884-900. <https://doi.org/10.1128/jb.172.2.884-900.1990>
15. Dao KHT, Hamer KE, Clark CL, Harshman LG. Pyoverdine production by *Pseudomonas aeruginosa* exposed to metals or an oxidative stress agent. *Ecol Appl*. 1999;9:441-8. [https://doi.org/10.1890/1051-0761\(1999\)009\[0441:PPBPAE\]2.0.CO;2](https://doi.org/10.1890/1051-0761(1999)009[0441:PPBPAE]2.0.CO;2)
16. Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A*. 2005;102:11076-81. <https://doi.org/10.1073/pnas.0504266102>
17. Thai VC, Lim TK, Le KPU, Lin Q, Nguyen TTH. iTRAQ-based proteome analysis of fluoroquinolone-resistant *Staphylococcus aureus*. *J Glob Antimicrob Resist*. 2017;8:82-9. <https://doi.org/10.1016/j.jgar.2016.11.003>
18. Zhou Y, Chen WN. iTRAQ-Coupled 2-D LC-MS/MS analysis of membrane protein profile in *Escherichia coli* incubated with apidaecin IB. *PLoS One*. 2011;6:e20442. <https://doi.org/10.1371/journal.pone.0020442>
19. Sanz-García F, Hernando-Amado S, Martínez JL. Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics. *Front Genet*. 2018;9:451. <https://doi.org/10.3389/fgene.2018.00451>
20. Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol*. 2003;52:403-8. <https://doi.org/10.1099/jmm.0.05132-0>
21. Kumar A, Lorand D. Robust $\Delta\Delta t$ estimate. *Genomics*. 202;113:420-7. <https://doi.org/10.1016/j.ygeno.2020.12.009>
22. Huynh TQ, Nguyen TTH. The effects of sub-MIC ciprofloxacin exposure on antibiotic susceptibility and virulence factors in *Pseudomonas aeruginosa* ATCC 9027. *Int J Life Sci Res Arch*. 2022;3:70-7. <https://doi.org/10.53771/ijlsra.2022.3.1.0075>
23. Sabnis A, Hagart KL, Klöckner A, et al. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. *eLife*. 2021;10:e65836. <https://doi.org/10.7554/eLife.65836>
24. Mortensen NP, Fowlkes JD, Sullivan CJ, et al. Effects of colistin on surface ultrastructure and nanomechanics of *Pseudomonas aeruginosa* cells. *Langmuir*. 2009;25:3728-33. <https://doi.org/10.1021/la803898g>
25. O'Driscoll NH, Cushnie TPT, Matthews KH, Lamb AJ. Colistin causes profound morphological alteration but minimal cytoplasmic membrane perforation in populations of *Escherichia coli* and *Pseudomonas aeruginosa*. *Arch Microbiol*. 2018;200:793-802. <https://doi.org/10.1007/s00203-018-1485-3>
26. Basan M, Zhu M, Dai X, et al. Inflating bacterial cells by increased protein synthesis. *Mol Syst Biol*. 2015;11:836. <https://doi.org/10.15252/msb.20156178>
27. Schaechter M, MaalØe O, Kjeldgaard NO. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella*

- typhimurium*. Microbiology. 1958;19:592-606. <https://doi.org/10.1099/00221287-19-3-592>
28. Seiffein NL, Ali GH. Effect of subinhibitory concentrations of selected antibiotics and propolis on pyocyanin and biofilm production among *Pseudomonas aeruginosa* isolates in Alexandria, Egypt. Egypt J Med Microbiol. 2021;30:129-37. <https://doi.org/10.21608/ejmm.2021.198932>
 29. Jones A, Elphick H, Pettitt E, Everard ML, Evans GS. Colistin stimulates the activity of neutrophil elastase and *Pseudomonas aeruginosa* elastase. Eur Respir J. 2002;19:1136-41. <https://doi.org/10.1183/09031936.02.00230602>
 30. Carretero-Ledesma M, Garcia-Quintanilla M, Martín-Peña R, Pulido MR, Pachón J, McConnell MJ. Phenotypic changes associated with colistin resistance due to lipopolysaccharide loss in *Acinetobacter baumannii*. Virulence. 2018;9:930-42. <https://doi.org/10.1080/21505594.2018.1460187>
 31. Cianciulli Sesso A, Lilić B, Amman F, Wolfinger MT, Sonnleitner E, Bläsi U. Gene expression profiling of *Pseudomonas aeruginosa* upon exposure to colistin and tobramycin. Front Microbiol. 2021;12:937. <https://doi.org/10.3389/fmicb.2021.626715>
 32. Vallet I, Diggle SP, Stacey RE, et al. Biofilm formation in *Pseudomonas aeruginosa*: fimbrial cup gene clusters are controlled by the transcriptional regulator MvaT. J Bacteriol. 2004;186:2880-90. <https://doi.org/10.1128/JB.186.9.2880-2890.2004>
 33. Xu H, Lin W, Xia H, et al. Influence of ptsP gene on pyocyanin production in *Pseudomonas aeruginosa*. FEMS Microbiol Lett. 2005;253:103-9. <https://doi.org/10.1016/j.femsle.2005.09.027>
 34. Kozak M. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol Rev. 1983;47:1-45. <https://doi.org/10.1128/mr.47.1.1-45.1983>
 35. Lipowska J, Miks CD, Kwon K, et al. Pyrimidine biosynthesis in pathogens - structures and analysis of dihydroorotases from *Yersinia pestis* and *Vibrio cholerae*. Int J Biol Macromol. 2019;136:1176-87. <https://doi.org/10.1016/j.ijbiomac.2019.05.149>
 36. UniProt. 2023. grpE - Protein GrpE - *Pseudomonas aeruginosa*. UniProtKB. Accessed on: 18 July 2023. Available at: <https://www.uniprot.org/uniprotkb/A0A069Q2K4/entry>
 37. Poole RK. Flavohaemoglobin: the pre-eminent nitric oxide-detoxifying machine of microorganisms. F1000Res. 2020;9:7. <https://doi.org/10.12688/f1000research.20563.1>
 38. Damron FH, Napper J, Teter MA, Yu HD. Lipotoxin F of *Pseudomonas aeruginosa* is an AlgU-dependent and alginate-independent outer membrane protein involved in resistance to oxidative stress and adhesion to A549 human lung epithelia. Microbiology (Reading). 2009;155:1028-38. <https://doi.org/10.1099/mic.0.025833-0>
 39. Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol Microbiol. 1999;34:305-16. <https://doi.org/10.1046/j.1365-2958.1999.01600.x>
 40. Lin YM, Wu SJ, Chang TW, et al. Outer membrane protein I of *Pseudomonas aeruginosa* is a target of cationic antimicrobial peptide/protein. J Biol Chem. 2010;285:8985-94. <https://doi.org/10.1074/jbc.M109.078725>
 41. Han ML, Velkov T, Zhu Y, et al. Polymyxin-induced lipid A deacylation in *Pseudomonas aeruginosa* perturbs polymyxin penetration and confers high-level resistance. ACS Chem Biol. 2018;13:121-30. <https://doi.org/10.1021/acscchembio.7b00836>
 42. Li H, Luo YF, Williams BJ, Blackwell TS, Xie CM. Structure and function of OprD protein in *Pseudomonas aeruginosa*: from antibiotic resistance to novel therapies. Int J Med Microbiol. 2012;302:63-8. <https://doi.org/10.1016/j.ijmm.2011.10.001>
 43. Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β -lactams in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2011;55:1211-21. <https://doi.org/10.1128/AAC.01252-10>
 44. Ghosh D, Veeraraghavan B, Elangovan R, Vivekanandan P. Antibiotic resistance and epigenetics: more to it than meets the eye. Antimicrob Agents Chemother. 2020;64:e02225-19. <https://doi.org/10.1128/AAC.02225-19>
 45. Riber L, Hansen LH. Epigenetic memories: the hidden drivers of bacterial persistence? Trends Microbiol. 2021;29:190-4. <https://doi.org/10.1016/j.tim.2020.12.005>
 46. Adam M, Murali B, Glenn NO, Potter SS. Epigenetic inheritance based evolution of antibiotic resistance in bacteria. BMC Evol Biol. 2008;8:52. <https://doi.org/10.1186/1471-2148-8-52>
 47. Ontong JC, Ozioma NF, Voravuthikunchai SP, Chusri S. Synergistic antibacterial effects of colistin in combination with aminoglycoside, carbapenems, cephalosporins, fluoroquinolones, tetracyclines, fosfomycin, and piperacillin on multidrug resistant *Klebsiella pneumoniae* isolates. PLoS One. 2021;16:e0244673. <https://doi.org/10.1371/journal.pone.0244673>
 48. Paul M, Daikos GL, Durante-Mangoni E, et al. Colistin alone versus colistin plus meropenem for treatment of severe infections caused by carbapenem-resistant Gram-negative bacteria: an open-label, randomised controlled trial. Lancet Infect Dis. 2018;18:391-400. [https://doi.org/10.1016/S1473-3099\(18\)30099-9](https://doi.org/10.1016/S1473-3099(18)30099-9)
 49. Kaye KS, Marchaim D, Thamlikitkul V, et al. Colistin monotherapy versus combination therapy for carbapenem-resistant organisms. NEJM Evid. 2022;2:EVIDoa2200131. <https://doi.org/10.1056/EVIDoa2200131>

50. Huang C, Chen I, Tang T. Colistin Monotherapy versus colistin plus meropenem combination therapy for the treatment of multidrug-resistant *Acinetobacter baumannii* infection: a meta-analysis. *J Clin Med*. 2022;11:3239. <https://doi.org/10.3390/jcm11113239>
51. Sanz-García F, Hernando-Amado S, Martínez JL. Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics. *Front Genet*. 2018;9:451. <https://doi.org/10.3389/fgene.2018.00451>
52. Savli H, Karadenizli A, Kolayli F, et al. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol*. 2003;52:403-8. <https://doi.org/10.1099/jmm.0.05132-0>
53. Tomás M, Doumith M, Warner M, et al. Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother*. 2010;54:2219-24. <https://doi.org/10.1128/AAC.00816-09>
54. Kumar A, Lorand D. Robust $\Delta\Delta\text{ct}$ estimate. *Genomics* 2021;113:420-7. <https://doi.org/10.1016/j.ygeno.2020.12.009>

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Table S1. Primers used in RT-qPCR.

Genes	Sequence (5'-3') (F: forward; R: reverse)	Size (bp)	Reference
<i>rplU</i>	F - CGCAGTGATTGTTACCGGTG R - AGGCCTGAATGCCGGTGATC	305	51
<i>rpoD</i>	F - GGGCGAAGAAGGAAATGGTC R - CAGGTGGCGTAGGTGGAGAA	178	52
<i>mvaT</i>	F - GCCTGAAGTCCCTGGAACAA R - TGCGGGTTCTTGACTGCTT	208	This study
<i>grpE</i>	F - GGATGGAAGTACCCTGAAG R - CGTTGAGCAGGTAGCCTTTC	180	This study
<i>oprD</i>	F- GGGCCGTTGAAGTCGGAGTA R- GGCGACAACGCGGCGAAGG	194	53
<i>hmp</i>	F - ATTTCTACCGCACCATGCTC R - GGAGACATGCTTGTTACCA	182	This study
<i>fur</i>	F - GATCCTGCAGATGCTCGACT R - GTATCGACGCAGACCATGTG	225	This study

Table S2. The antibiotic susceptibility profiles of *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2. Tested antibiotics included meropenem (Me), tobramycin (Tb), ceftazidime (Cz), gentamicin (Ge), ciprofloxacin (Ci), cefepime (Cm), piperacillin/ tazobactam (Pt) and imipenem (Im) (NAM KHOA Biotek Company, Vietnam). The susceptibility classification: susceptible (S), intermediate (I), resistant (R), was based on the CLSI 2022 and CLSI 2023 guidelines.

Antibiotics	<i>P. aeruginosa</i> ATCC 9027	Col-E1	Col-E2
Me (10 µg)	S	S	S
Tb (10 µg)	S	S	S
Cz (30 µg)	S	S	S
Ge (10 µg)	S	S	S
Ci (5 µg)	S	S	S
Cm (30 µg)	S	S	S
Pt (100/10 µg)	S	S	S
Im (10 µg)	S	S	S

Table S3. Proteins of *P. aeruginosa* persisting in the up/downregulated expression after stopping exposure to colistin.

Accession no.	Name	Col-E1	Col-E2
Q9I4D2	Uncharacterized protein PA1206	17.94731	27.79713
Q9HV42	Protein GrpE	14.92797	11.22015
Q9I687	Methylenetetrahydrofolate reductase MetF	14.32188	9.246985
Q9I5T1	Ribulose-phosphate 3-epimerase Rpe	13.67728	3.483353
Q9HU11	Uncharacterized protein PA5178	13.61444	17.8649
Q9I0H4	Flavohemoprotein Hmp	12.07815	19.31965
Q9HWC7	50S ribosomal protein L10 RplJ	10.04614	10.42317
Q9HZK2	Peptidoglycan-binding protein CsiV PA3003	8.994982	10.23297
Q9HUD0	50S ribosomal protein L31 RpmE	8.912523	13.61446
Q9I649	Uncharacterized protein PA0469	8.74982	10.09252
Q9HWC8	50S ribosomal protein L7/L12 RplL	8.203513	6.606906
O82853	Ribosome-recycling factor Frr	8.128324	8.669641
sp Q9HWD1	30S ribosomal protein S7 RpsG	6.760828	5.597592
sp P30819	Nicotinate-nucleotide pyrophosphorylase [carboxylating] NadC	6.546321	5.058208
sp Q9LCT6	D-alanine~D-alanine ligase B DdlB	6.426878	4.764332
tr Q9HVX4	Uncharacterized protein PA4441	6.251739	5.296658
P11221	Major outer membrane lipoprotein OprI	6.194435	6.823343
Q9I4D6	Probable hydrolase PA1202	5.888465	6.606948
O52761	50S ribosomal protein L17 RplQ	5.834479	4.897795
Q9HV58	30S ribosomal protein S15 RpsO	5.727933	5.223946
O82850	30S ribosomal protein S2 RpsB	5.546279	6.053377
P50587	Orotate phosphoribosyltransferase PyrE	5.495409	3.83712
Q9HWD9	30S ribosomal protein S19 RpsS	5.370351	4.6132
P0C2B2	Thiol:disulfide interchange protein DsbA	5.34566	5.058212
Q9HYY5	Uncharacterized protein PA3255	5.152282	4.405538
Q9I0R1	Nitroreductase domain-containing protein PA2575	4.965929	4.808387
Q9HTD0	Biotin carboxylase PA5436	4.943103	2.290886
Q9I5V8	30S ribosomal protein S21 RpsU	4.852877	2.167708

Accession no.	Name	Col-E1	Col-E2
P05384	DNA-binding protein HU-beta HupB	4.786308	3.515589
Q9I6H4	FAD-binding PCMH-type domain-containing protein PA0317	4.487447	3.296125
Q9HWF4	50S ribosomal protein L15 RplO	4.285465	4.055078
Q9HWE7	50S ribosomal protein L5 RplE	4.265793	3.630787
Q9I0H1	Uncharacterized protein PA2667	4.207239	3.749739
Q9HTQ6	Xanthine phosphoribosyltransferase Xpt	4.092599	6.516263
Q9HWD7	50S ribosomal protein L23 RplW	4.03648	4.226703
Q9HVM1	30S ribosomal protein S20 RpsT	3.999455	4.875294
Q9HUM1	GTPase HflX	3.981078	4.466839
P29436	50S ribosomal protein L34 RpmH	3.890491	3.435578
Q9HWD4	30S ribosomal protein S10 RpsJ	3.837092	3.53186
P43501	Protein PilH	3.784442	3.597531
Q9HX24	Soluble lytic transglycosylase B SltB1	3.681288	4.16872
Q9HXP8	Signal recognition particle protein Ffh	3.630795	2.500361
Q9I3D2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex SucB	3.580982	2.870779
O82851	Elongation factor Ts Tsf	3.580958	2.666865
Q03456	Ferric uptake regulation protein Fur	3.531804	3.235966
Q9I2T8	Peptidyl-prolyl cis-trans isomerase D PpiD	3.499456	3.499489
Q9HWC4	Transcription termination/antitermination protein NusG	3.435607	2.157781
Q9HXU8	Lipotoxin F LptF	3.388423	3.2063
Q9HZU7	Probable outer membrane protein PA2900	3.357389	2.81841
Q9HXJ6	Ancillary SecYEG translocon subunit PA3801	3.296115	2.779725
Q9HYQ4	Probable transcriptional regulator PA3341	3.265904	3.296134
Q9HWE2	50S ribosomal protein L16 RplP	3.20627	2.679142
Q9HX68	Two-component response regulator RocA1	3.191534	2.387775
Q9HT16	ATP synthase subunit b AtpF	3.04791	2.098944
Q9HXQ2	50S ribosomal protein L19 RplS	3.033923	2.535111
Q9HVV2	50S ribosomal protein L13 RplM	2.9648	2.703956
O54438	3-oxoacyl-[acyl-carrier-protein] reductase FabG	2.937662	2.089303
P33641	Outer membrane protein assembly factor BamD	2.91075	2.535151
Q9HXU0	Lysine-tRNA ligase LysS	2.910745	2.157754
Q9I352	Bacteriohemerythrin PA1673	2.884056	2.897319
Q60169	Porphobilinogen deaminase HemC	2.870774	2.25941
Q9HT14	ATP synthase subunit a AtpB	2.80543	3.64756
Q9I4F9	Two-component response regulator PhoP	2.558585	2.409877
Q9I5Z9	Uncharacterized protein PA0537	2.488858	2.249018
Q9HV72	Poly(A) polymerase I PcnB	2.466055	2.69154
Q51344	Aspartate-semialdehyde dehydrogenase Asd	2.421053	2.5704
Q9HTV1	Transcription termination factor Rho	2.409909	2.691538
Q9HW86	MvaT	2.376879	2.098937
P37799	Biotin carboxyl carrier protein of acetyl-CoA carboxylase AccB	2.322744	2.023015
Q9I6W7	Uncharacterized protein PA0165	2.322728	2.208027
Q9HWZ6	Inorganic pyrophosphatase Ppa	2.322698	2.729

Accession no.	Name	Col-E1	Col-E2
Q9HUP3	NH(3)-dependent NAD(+) synthetase NadE	2.228458	2.032393
P48247	Glutamate-1-semialdehyde 2,1-aminomutase HemL	2.208013	2.051177
Q9I640	Probable N-acetyltransferase PA0478	2.197862	2.951235
P55222	cAMP-activated global transcriptional regulator Vfr	2.177672	2.511875
Q9HTI6	OpuAC domain-containing protein PA5378	2.167739	2.098964
Q9HYC7	Methionine-tRNA ligase MetG	2.098967	2.249083
Q9I6A8	dITP/XTP pyrophosphatase PA0387	2.089273	2.177703
Q9I4D4	NAD(P)H-dependent FMN reductase PA1204	2.07968	2.365936
Q9HY79	Bacterioferritin BfrB	2.079679	2.779697
G3XCV0	Transcriptional regulator FleQ	2.070149	2.964863
Q9I140	Glycine cleavage system T protein GcvT2	2.013718	2.089333
Q9HY15	Uncharacterized protein PA3611	-3.14797	-2.7545
P72151	B-type flagellin FliC	-3.0621	-2.59398
Q9HYZ7	Probable septum site-determining protein MinC	-2.8314	-4.11068
Q9I083	Probable outer membrane protein PA2760	-2.4662	-2.09897
Q9I762	Gly-zipper_Omp domain-containing protein PA0070	-2.43194	-4.20801
Q9HXS5	Uncharacterized protein OS= <i>Pseudomonas aeruginosa</i> PA3716	-2.36582	-2.53511
G3XCX7	Type IV pilus inner membrane component PilP	-2.35512	-2.30148
O68562	Outer membrane protein assembly factor BamE	-2.33353	-2.47747
P30720	10 kDa chaperonin GroS	-2.23881	-2.85767
Q9I671	Glutaryl-CoA dehydrogenase GcdH	-2.15765	-2.43244
Q9I456	Probable outer membrane protein PA1288	-2.1479	-2.17772
Q9HTE9	Serine hydroxymethyltransferase 1 GlyA1	-2.01373	-6.33983

Table S4. Proteins of *P. aeruginosa* turning back to normal expression after stopping exposure to colistin.

Accession No.	Name	Col-E1	Col-E2
Q9HYJ2	Uncharacterized protein PA3413	5.888439	1.527572
Q9HVV7	Large-conductance mechanosensitive channel MscL	3.630763	1.828104
Q9I0K8	JmjC domain-containing protein PA2630	3.614107	1.577618
Q9HX76	Probable DNA binding protein PA3940	3.548165	-1.16413
Q9HUM9	30S ribosomal protein S6 RpsF	3.235949	1.995252
Q9HXZ2	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha AccA	3.221083	1.802995
Q9HVF9	Peptidase_M48 domain-containing protein PA4632	2.83137	1.288235
Q9I794	Uncharacterized protein PA0038	2.741586	-3.09127
Q9HVD1	Lipid A deacylase PagL	2.642393	-1.02803
Q9HVF1	Probable malate:quinone oxidoreductase 2 Mqo2	2.642364	1.606944
Q9HXP9	30S ribosomal protein RpsP	2.630257	1.931984
Q9I3D8	Uncharacterized protein PA1579	2.606149	1.729821
Q9I6F1	Thymidylate synthase ThyA	2.523474	1.004644
Q9HXY5	Skp-like protein PA3647	2.500337	1.84504
Q9I7C5	Chromosomal replication initiator protein DnaA	2.477398	1.729855
Q9HVV8	Stringent starvation protein B SspB	2.398827	1.896704
Q9HUG5	Alpha-1,3-rhamnosyltransferase WapR	2.365945	1.914278
Q9I6I0	Probable acyl-CoA dehydrogenase PA0508	2.290872	1.061713
Q9HWW7	Probable thioredoxin PA4061	2.28035	1.432194
Q9HUF1	Peptide methionine sulfoxide reductase MsrA	2.167708	1.541717
Q9HVV4	Ubiquinol-cytochrome c reductase iron-sulfur subunit PA4431	2.167699	1.67495
Q9HV34	Polyamine aminopropyltransferase 2 SpeE2	2.157744	1.137626
Q9HZA7	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta AccD	2.137927	1.563151
Q9HTJ1	NAD/NADP-dependent betaine aldehyde dehydrogenase BetB	2.118328	1.191281
Q9I047	Transaldolase Tal	2.079721	1.479115
Q9HTC2	Probable coenzyme A transferase PA5445	2.022982	1.241629
P32722	Porin D OprD	-8.47278	-1.94094
Q51551	Dihydroorotase-like protein PyrC'	-3.1477	-1.57029
Q9I3I6	Chromosome partition protein Smc	-2.74194	-1.40604
Q9I0M2	Thioredoxin reductase TrxB1	-2.6792	-1.49963
Q9I576	4-hydroxyphenylpyruvate dioxygenase Hpd	-2.59411	-1.44545
O54439	Acyl carrier protein 1 AcpP1	-2.36582	-1.63675
Q9HX32	LPS-assembly lipoprotein LptE	-2.34408	-1.61428
Q9X6W6	Bifunctional protein PyrR	-2.33339	-1.45885
Q9HYT2	Probable 3-hydroxyisobutyrate dehydrogenase PA3312	-2.28036	-1.16951
Q9HXI6	Serine O-acetyltransferase CysE	-2.18779	-1.83665
Q9HUN4	Alanine racemase, biosynthetic Alr	-2.15779	-1.49972
Q9HXX6	Uncharacterized protein PA3674	-2.09904	-1.10665
Q9HXX9	Queuine tRNA-ribosyltransferase Tgt	-2.09892	-1.42565
Q9I5A7	OmpA-like domain-containing protein PA0833	-2.05125	-1.5995
Q9I6H6	DUF4399 domain-containing protein PA0315	-2.02298	-1.6749

Table S5. Calculation of fold change and confidence level 95% CI (error bar) according to the study of Kumar and Lorand 2021⁵⁴

Genes	$\Delta\Delta C_t$	Standard deviation	CI 95%	Fold change ($2^{-\Delta\Delta C_t}$)	95%CI of fold change
<i>grpE</i>	-0.34	0.64	(-0.89, 0.22)	1.26	(0.86, 1.85)
<i>fur</i>	-0.99	1.98	(-2.68, 0.68)	2.00	(0.62, 6.41)
<i>oprD</i>	0.82	0.21	(-0.25, 1.89)	0.57	(0.27, 1.19)
<i>hmp</i>	-0.38	1.74	(-3.64, 2.88)	1.30	(0.28, 12.49)
<i>mvaT</i>	-0.07	0.76	(-0.74, 0.60)	1.05	(0.66, 1.67)

Table S6. Comparison of protein regulation fold change and RNA fold change of Col-E1 against *P. aeruginosa* ATCC 9027. Fold change normalization: fold change that was smaller than 1 was represented as negative value (-1/fold change).

Genes	Protein expression fold change	mRNA fold change
<i>grpE</i>	14.93	1.26
<i>fur</i>	3.53	2.00
<i>oprD</i>	-8.47	-1.75
<i>hmp</i>	12.08	1.30
<i>mvaT</i>	2.38	1.05

Table S7. The biological process (Gene ontology) of 22 clusters of DEPs of *P. aeruginosa* after being exposed to colistin. The clusters were generated by using the MCL clustering option STRING 11.5 (<https://string-db.org/>).

Cluster ID	Gene count	Biological process (gene ontology)
1	25	Translation Gene expression Cellular protein metabolic process Cellular macromolecule biosynthetic process Cellular nitrogen compound biosynthetic process
2	7	Tetrahydrofolate interconversion Glycine metabolic process L-serine metabolic process Serine family amino acid biosynthetic process Serine family amino acid metabolic process
3	5	Pyrimidine biosynthesis
4	5	ATP synthesis coupled proton transport
5	5	malonyl-CoA biosynthetic process acyl-CoA metabolic process Fatty acid biosynthetic process Fatty acid metabolic process Sulfur compound biosynthetic process
6	3	Citrate cycle Pyruvate metabolism Microbial metabolism in diverse environments
7	3	Protein folding Stress response
8	3	NAD biosynthetic process

Cluster ID	Gene count	Biological process (gene ontology)
9	2	DNA replication Chromosome condensation
10	2	GMP biosynthetic process, and XMP salvage
11	2	Queuosine biosynthetic process
12	2	acyl-CoA metabolic process
13	2	Carbohydrate metabolic process
14	2	Peptidoglycan catabolic process
15	2	Fatty acid elongation
16	2	Fatty acid biosynthetic process
17	2	Polyamine biosynthetic process Spermidine biosynthetic process
18	2	Peptidoglycan synthesis Cell wall biogenesis/degradation
19	2	tRNA aminoacylation for protein translation
20	2	Protoporphyrinogen IX biosynthetic process
21	2	DNA-templated transcription termination
22	2	Gram-negative-bacterium-type cell outer membrane assembly Protein insertion into membrane

Table S8. Quantity and quality of extracted RNA from *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2.

Strains	RNA concentration (ng/μL)	Ratio 260/280
<i>P. aeruginosa</i> ATCC 9027	845.82	2.14
Col-E1	403.91	2.03
Col-E2	650.66	2.00

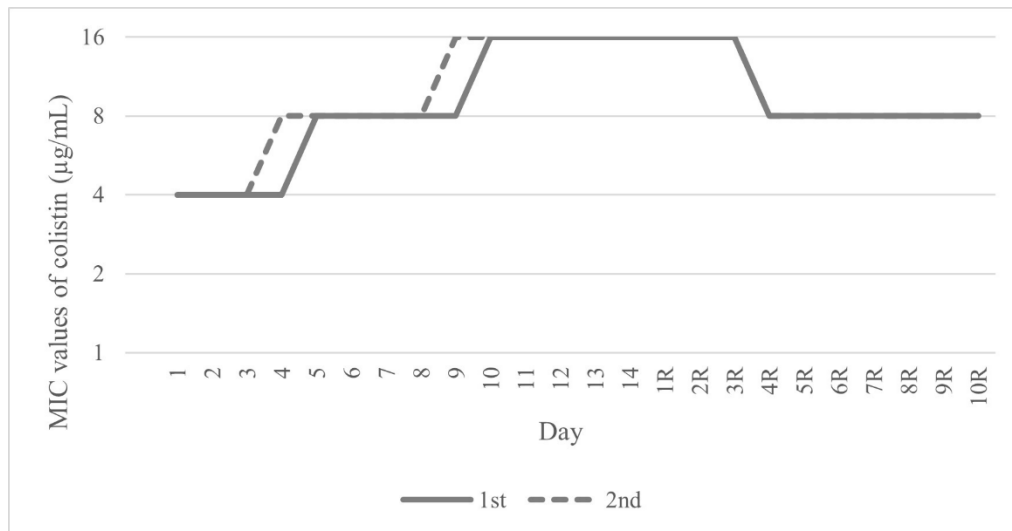


Figure S1. The colistin MIC values of *P. aeruginosa* over the exposure. Day 1, day 14, and day 10R indicated the MIC value of *P. aeruginosa* ATCC 9027, Col-E1, and Col-E2, respectively. Two biological replications were performed. Solid line: 1st replication, dashed line: 2nd replication.

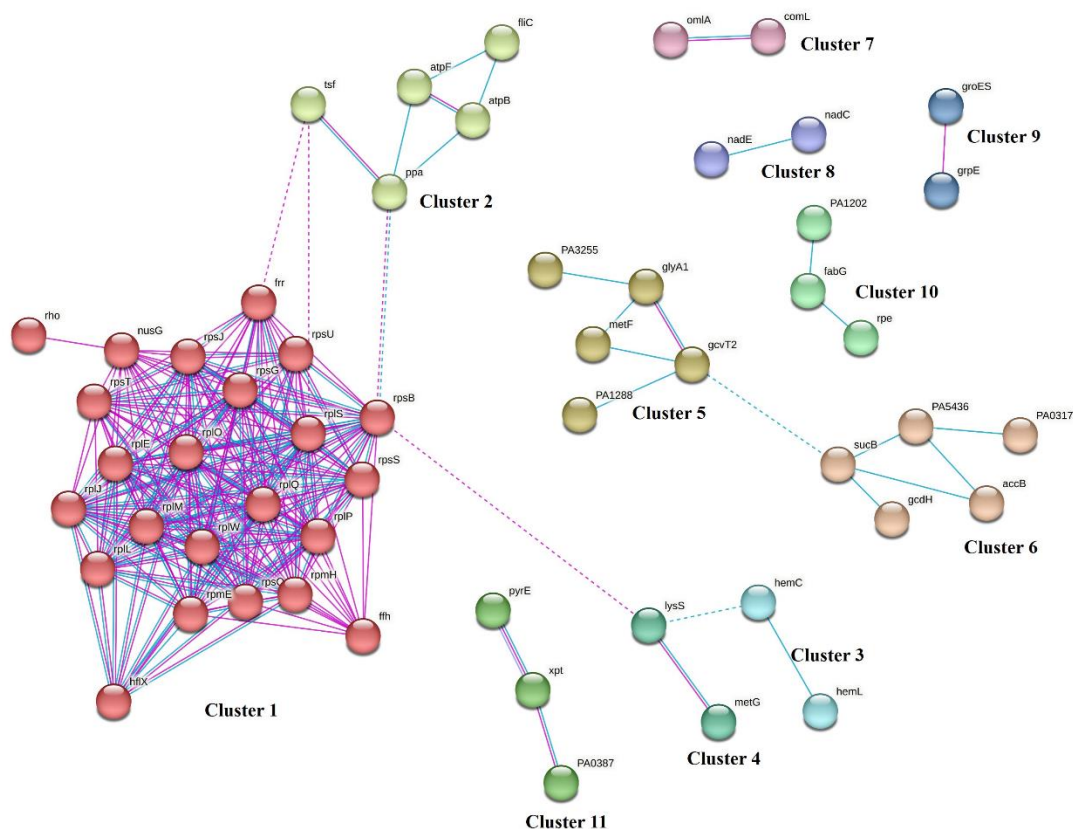


Figure S2. The interactions between *P. aeruginosa* proteins kept a differentially expressed trend in both Col-E1 and Col-E2. The disconnected proteins were hidden. The interactions were performed by STRING 11.5 (<https://string-db.org/>). The STRING showed some proteins with different names compared to the Uniprot platform, including OmlA corresponding to BamE and ComL corresponding to BamD. The DEPs were grouped into 11 clusters by STRING 11.5 using the MCL clustering option. Cluster 1: DNA-templated transcription and termination, translation, gene expression, cellular macromolecule biosynthetic process, cellular nitrogen compound biosynthetic process; Cluster 2: ATP synthesis coupled proton transport; Cluster 3: Protoporphyrinogen IX biosynthetic process; Cluster 4: tRNA aminoacylation for protein translation; Cluster 5: Tetrahydrofolate interconversion, glycine metabolic process, L-serine metabolic process, serine family amino acid metabolic process, alpha-amino acid biosynthetic process; Cluster 6: acyl-CoA biosynthetic process, acyl-CoA metabolic process, fatty acid biosynthetic process, fatty acid metabolic process, carboxylic acid catabolic process; Cluster 7: Protein insertion into membrane, gram-negative-bacterium-type cell outer membrane assembly; Cluster 8: NAD biosynthetic process; Cluster 9: Protein folding, stress response; Cluster 10: Carbohydrate metabolism, fatty acid elongation; and Cluster 11: Nucleobase-containing small molecule biosynthetic process, ribonucleoside monophosphate biosynthetic process, nucleoside metabolic process, nucleotide metabolic process, nucleobase-containing compound biosynthetic process.

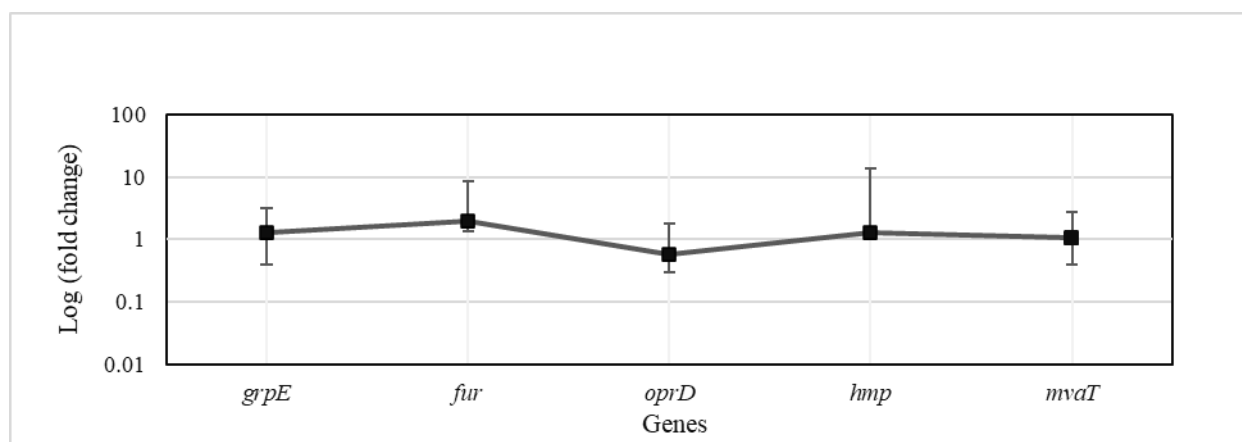


Figure S4. RT-qPCR analysis of selected genes in *P. aeruginosa* after exposure to colistin. Fold change was visualized on a log scale. Gene expression of *P. aeruginosa* ATCC 9027 was 1. Up-regulated genes were defined as values greater than 1 and down-regulated when values were smaller than 1. Fold change and confidence level 95% CI (error bar) were calculated according to the study of Kumar and Lorand 2021.⁵⁴