CHANGES IN BACTERIAL FITNESS DURING THE *PSEUDOMONAS AERUGINOSA* EXPERIMENTAL ADAPTATION TO COLISTIN

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Pseudomonas aeruginosa, the opportunistic pathogen, occupies one of the leading places in the structure of pathogens causing nosocomial infections, which is due to high adaptive potential and the ability to quickly develop antimicrobial resistance. The study aimed to assess the influence of the *P. aeruginosa* adaptation to colistin on bacterial fitness. A total of nine isolates obtained during the experimental evolution of the *P. aeruginosa* strain (laboratory number 1202) under conditions of increasing colistin concentrations, the growth kinetics of which was compared to that of wild type strain, were included in the study; the whole genome sequencing of all isolates was performed, and the minimum inhibitory concentration of colistin was determined. Growth rate was estimated using the Varioskan LUX multimodal reader (Thermo Scientific, USA) throughout 18 h at 37 °C; optical density (OD) at λ = 600 nm was measured every 15 min. The maximum growth rate (GR_{max}, i.e. the maximum change in OD within 1h) and the time to reach 50% of the maximum OD reported when growing the wild type *Pa_1202_0* strain ($T\_OD_{50\%}$) were considered. Isolates of the clone carrying mutations of the genes *phoQ*, *lptA*, and *prs* showed low fitness values compared to wild type strains. For example, GR_{max} of the isolate *Pa_1202_43* was 0.029 OD/h vs. 0.182 OD/h reported for the original isolate *Pa_1202_0*, and it reached $OD_{50\%}$ 4.6 h later. The growth characteristics of the clone carrying mutations of *lpxL* and *lptB*, as well as the clone carrying mutant *pmrB* were generally comparable with the characteristics of the wild type strain. Thus, the genome modifications observed during the *P. aeruginosa* adaptation to colistin have an ambiguous effect on bacterial fitness.

Keywords: *Pseudomonas aeruginosa*, nosocomial infections, bacterial fitness, colistin, resistance genes

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ИЗМЕНЕНИЕ БАКТЕРИАЛЬНОГО ФИТНЕСА В ХОДЕ ЭКСПЕРИМЕНТАЛЬНОЙ АДАПТАЦИИ *PSEUDOMONAS AERUGINOSA* К КОЛИСТИНУ

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Оппортунистический патоген *Pseudomonas aeruginosa* занимает одно из ведущих мест в структуре возбудителей нозокомиальных инфекций, что связано с высоким адаптивным потенциалом и способностью быстро формировать устойчивость к антимикробным препаратам. Целью работы было оценить влияние адаптации *P. aeruginosa* к колистину на бактериальный фитнес. В исследование включили 9 изолятов, полученных в ходе экспериментальной эволюции штамма *P. aeruginosa* (лабораторный номер 1202) в условиях возрастающей концентрации колистина, кинетику роста которых сравнивали с родительским штаммом; у всех изолятов провели полногеномное секвенирование и определили минимальную подавляющую концентрацию колистина. Темпы роста оценивали при помощи многофункционального ридера Varioskan LUX (Thermo Scientific, США) в течение 18 ч при 37 °C, каждые 15 мин измеряя оптическую плотность (ОП) при λ = 600 нм. Учитывали максимальную скорость роста ($СР_{max,}$ т. е. максимальное изменение ОП в течение 1 ч) и время, необходимое для достижения 50% от максимальной ОП, зарегистрированной при росте родительского штамма *Pa_1202_0* (Т_ОП50%). Изоляты клона с мутациями в генах *phoQ*, *lptA* и *prs* отличались низкими показателями фитнеса от родительских штаммов. Например, $СР_{max}$ изолята *Pa_1202_43* составила 0,029 ОП/ч против 0,182 ОП/ч у исходного изолята *Pa_1202_0*, а $ОП_{50\%}$ он достигал на 4,6 ч позже. Ростовые характеристики клона с мутациями в *lpxL* и *lptB*, а также клона, несущего мутированный *pmrB*, в целом были сопоставимы с показателями родительского штамма. Таким образом, модификации генома, наблюдавшиеся в ходе адаптации *P. aeruginosa* к колистину, оказывают неоднозначное влияние на бактериальный фитнес.

Ключевые слова: синегнойная палочка, нозокомиальные инфекции, бактериальный фитнес, колистин, гены резистентности

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Pseudomonas aeruginosa is an important opportunistic pathogen, the successful survival of which in clinical settings results from high adaptive potential. Quick adaptation to new ecological loci, antimicrobial drugs, and the immune system effectors allows *P. aeruginosa* to be one of the main causes of nosocomial morbidity [1]. The infections caused by multidrug-resistant *P. aeruginosa* strains are difficult to treat, and only a few antimicrobial drugs remain active against such pathogens. Colistin, the polymyxin antibiotic, is one of the “last chance” antibiotics [2].

The increasing clinical use of colistin inevitably leads to colistin resistance. Resistance to colistin is associated with the structural modification of its target, lipopolysaccharide (LPS), which decreases the antibiotic binding to the bacterial cell wall [3]. LPS modification and colistin resistance in *P. aeruginosa* are usually associated with damage to the

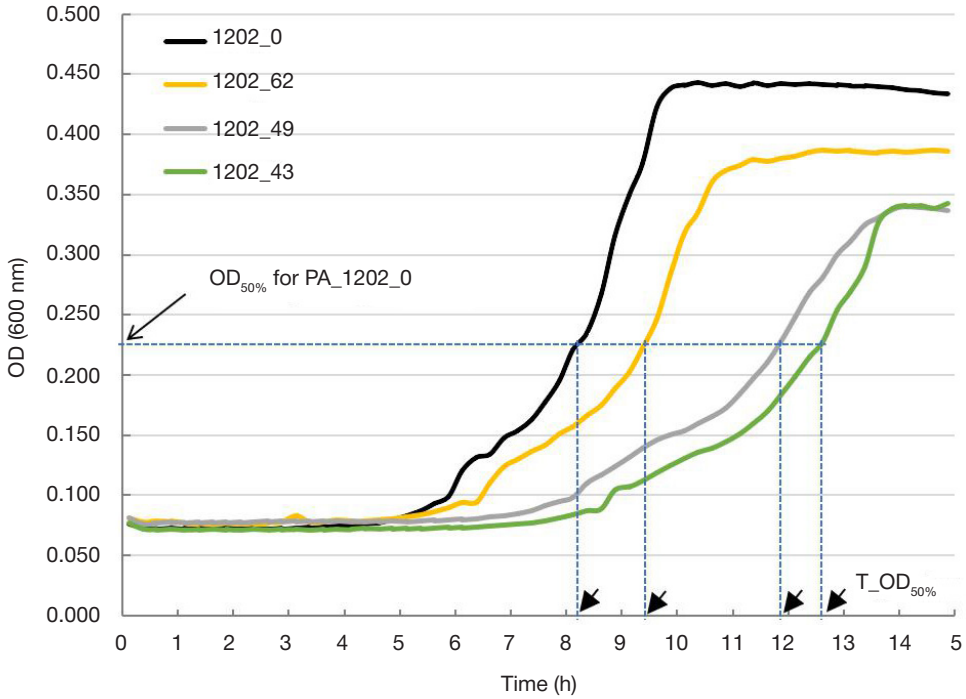

Fig. Growth curves of the wild type *Pa_1202_0* strain and descendants strains of the clone *Pa_phoQ/lptA/prs*. Bacteria were incubated in the 96-well plate at 37 °C, and optical density (OD) at λ = 600 nm was measured every 15 min. The *long open arrow* points to the OD value corresponding to 50% of the maximum OD reported when growing the wild type *Pa_1202_0* strain ($OD_{50\%}$). The *short closed arrows* point to the time needed by the studied isolates to reach $OD_{50\%}$ ($T\_OD_{50\%}$). The isolate characteristics are provided in the Table. Representative results of one of three repetitions of the experiment

PhoP-PhoQ and PmrA-PmrB two-component systems resulting from mutations of appropriate genes, although are not limited to these mechanisms [3, 4]. Mutations that form resistance are advantageous for carriers of mutations in the presence of antibiotic. However, the same mutations can reduce viability of microorganism as a whole, making it uncompetitive in the absence of antibiotic. Mutations that trigger alternative metabolic pathways in the cell and replace missing metabolism links can compensate for biological expenditures associated with resistance [5–7]. In this regard, assessment of bacterial fitness, i.e. viability level that can be inter alia expressed as the bacterial population growth rate changes [8], is an important complement to genetic analysis of the resistance mechanisms.

In the recent study focused on the *P. aeruginosa* experimental adaptation to colistin, we have shown that the genome evolved by alternative routes not only in different strains, but also within the same bacterial strain when developing colistin resistance [9]. Isolates of one experimental *P. aeruginosa* strain obtained at various stages of adaptation to colistin and analyzed by whole genome sequencing were used in this study. Isolates with various mutations were selected for the study, in which the growth kinetics was assessed and compared with that of the parent strain. The study aimed to investigate the relationship between genotypic and phenotypic characteristics in the experimental models, which highlights the importance of genetic background for the development of antimicrobial resistance, makes it possible to gain new knowledge about the mechanisms underlying antibiotic resistance, and outline new ways to overcome drug resistance of bacteria.

METHODS

We studied the *P. aeruginosa* strain (laboratory number 1202, genome deposited in GenBank) isolated from the environment in 2016 that was susceptible to all antibiotics and its *Pa_1202*-descendants isolates obtained during experimental adaptation to colistin, the methodology of which had been earlier discussed in detail [9].

To estimate bacterial fitness, we compared growth rates of the wild type and descendants *Pa_1202* strains obtained in the adaptive experiment. A single colony of the 24-h culture of each isolate was used to prepare a bacterial suspension, which was standardized by optical density to 0.5 McFarland units. A total of 10 mL of the Luria–Bertani broth were inoculated with 10 µL of the resulting suspension, after which 200 µL were collected and transferred to the well of the flat-bottom 96-well plate. The plate was sealed with the transparent film and incubated in the Varioskan LUX multimodal reader (Thermo Scientific, USA) for 18 h at 37 °C; optical density (OD) at λ = 600 nm was measured every 15 min. Growth curves were analyzed using the SkanIt v. 7.0 software tool (Thermo Scientific; USA). Growth rate was estimated based on two indicators: 1) maximum growth rate (GRmax, corresponds to the maximum change in OD within 1h measured in OD/h); 2) time to reach 50% of the maximum OD reported when growing the wild type *Pa_1202_0* strain ($T\_OD_{50\%}$) (Fig). The decrease in GRmax and the increase in $T\_OD_{50\%}$ were considered as the fitness decrease. The experiment was carried out in triplicate.

The minimum inhibitory concentration (MIC) of colistin within the range of 0.25–16 mg/L was determined using the ComASP Colistin 0.25–16 kits (Liofilchem srl.; Italy), while higher MICs (up to 64 mg/L) were estimated by the broth microdilution method. The MIC values were interpreted based on their experimental dynamics, not clinical significance.

The whole genome sequencing was performed using the bacterial DNA extracted from the 24-h cultures of the experimental *Pa_1202* isolates grown from frozen samples (see above) on the Mueller–Hinton agar. The whole genome sequencing and bioinformatics analysis procedure has been earlier discussed in detail [9].

Statistical analysis was performed using the IBM SPSS Statistics v. 27.0 software (USA). The quantitative results are presented in the text and the Table as mean values (standard deviations). The Mann–Whitney U test was used to compare the GR_{max} and $T\_OD_{50\%}$ values; the differences were considered significant at $p < 0.05$.

Table. Phenotype and genotype of the isolates obtained during experimental adaptation to colistin

Isolate	Day	MIC of colistin (mg/L)	GR_{max} (OD/h)	$T\_OD_{50\%}$ (h)	*phoQ*	*pmrB*	*lpxL*	*lptA*	*lptB*	*prs*	*speE*	*hp/PA2117*	*tetC*	*oprH*
			Mean (SD)											
1202_0	0	1	0.182 (0.018)	8.8 (0.1)										
Clone *Pa_phoQ/lptA/prs*														
1202_43	11	32	0.029 (0.001) *	13.4 (0.2) *										
1202_49	13	1	0.038 (0.009) *	12.7 (0.2) *										
1202_62	16	2	0.140 (0.012)	10.0 (0.1) *										
Clone *Pa_phoQ/lpxL/lptB*														
1202_63	16	16	0.285 (0.015) *	8.9 (0.0)										
1202_80	20	2	0.268 (0.059)	9.0 (0.1)										
1202_95	28	16	0.163 (0.016) *	9.2 (0.1)										
Clone *Pa_pmrB*														
1202_37	9	1	0.155 (0.016)	8.9 (0.0)										
1202_44	11	2	0.219 (0.029)	9.0 (0.1)										
1202_88	24	16	0.198 (0.026)	7.9 (0.1) *										

Note: 10 *Pa_1202* isolates were obtained on the specified days of the experiment [9]. We determined the minimum inhibitory concentration (MIC) of colistin and assessed fitness by analysis of the growth curves and measurement of the maximum growth rate (GR_{max}) and the time to reach 50% of the maximum optical density (OD) reported when growing the wild type *Pa_1202_0* strain ($T\_OD_{50\%}$) (see Fig). Genes of the core genome were studied using whole genome sequencing. *Green* cells correspond to the gene sequences identical to *Pa_1202_0*, mutations are highlighted in *red*. Names of the genes involved in lipopolysaccharide synthesis and associated with colistin resistance are highlighted in *orange*; names of the genes of general metabolism not directly associated with colistin resistance are uncolored. SD — standard deviation. * — $p < 0.05$, comparison with *Pa_1202_0*.

RESULTS

Growth rates of the wild type *Pa_1202_0* strain and nine *Pa_1202* isolates representing three earlier described major clonal lineages obtained during experimental adaptation to colistin were assessed [9] (Table). Two clones carried the same mutation of *phoQ* (ins-ATCGCCT-1086), but were distinguished by mutations of other genes. In one case further damage was found in the genes *lptA* (ins-CCGCGC-490) and *prs* (T143→C), the clone was named *Pa_phoQ/lptA/prs*. In another case the *lpxL* (ins-C-335) and *lptB* (ins-GCG-27) genes were altered, the clone was named *Pa_phoQ/lpxL/lptB*. The third clone was characterized by mutation of the gene *pmrB* (T92→G) (the clone was named *Pa_pmrB*) combined with the damaged gene *hp/PA2117* (G326→A).

Isolates of the clone *Pa_phoQ/lptA/prs* showed low fitness compared to the wild type *Pa_1202_0* strain (Fig, Table). For example, GR_{max} of the isolate 1202_43 was 0.029 (0.001) OD/h vs. 0.182 (0.018) OD/h reported for the original *Pa_1202_0* isolate, and it reached $OD_{50\%}$ 4.6 h later.

The growth characteristics of the clones *Pa_ lpxL/lptB* and *Pa_pmrB* were generally comparable with the characteristics of the wild type *Pa_1202_0* strain (Table). Two isolates showed a significant increase in GR_{max} (1202_63) and a significant decrease in $T\_OD_{50\%}$ (1202_88), which suggested better growth rate compared to the wild type strain, despite the 16-fold increase in the colistin MIC (Table). Isolate 1202_95 of the clone *Pa_pmrB* showed a significantly decreased GR_{max}, however, the difference in $T\_OD_{50\%}$ from the original strain was non-significant.

DISCUSSION

In this study we have shown how the *P. aeruginosa* experimental adaptation affects bacterial fitness by assessing growth kinetics of the isolates with various genotypes. It is the most logical choice to explain the differences in fitness between representatives of three studied clones via analysis of the profiles of the genomic alterations typical for each clone. The genomes of isolates of the clone *Pa_phoQ/lptA/prs* comprise alterations of two types: 1) mutations of the genes *phoQ* and *lptA* that directly control biosynthesis of LPS, the main target of polymyxins [10, 11]; 2) mutations of the gene encoding ribose-phosphate pyrophosphokinase (prs) that is not directly associated with the LPS synthesis and controls the nucleotide synthesis and metabolism. PhoP, the component of the PhoPQ regulatory system, is directly involved in the LPS synthesis regulation, and its breakage is considered to be the common cause of colistin resistance [10]. The *lptA* gene product ensures the LPS assembly and outer membrane translocation [11]. In the clone *Pa_phoQ/lptA/prs*, the complex genomic alterations were combined with the most pronounced bacterial fitness decrease.

In the clone *Pa_phoQ/lpxL/lptB*, we found only mutations of the LPS synthesis genes, including the abovementioned *phoQ*, *lpxL* (gene encoding lauroyl acyltransferase ensuring the lipid A biosynthesis), and *lptB* (gene encoding the LptB2FG transporter transferring LPS to the outer membrane) [12, 13].

The *Pa_pmrB* clone combined mutations of the genes encoding the sensor kinase (pmrB) and the hypothetic protein (*hp/PA2117*). The PmrB kinase is a component of the two-component system ensuring regulation of multiple functions, including expression of the LPS operon genes; earlier it had been proven that damage to the *pmrB* gene decreases the *P. aeruginosa* susceptibility to polymyxins [14, 15]. To date, the *hp/PA2117* gene product has not been verified.

CONCLUSIONS

Thus, the genome modifications observed during the *P. aeruginosa* adaptation to colistin have an ambiguous effect on bacterial fitness. It is clear that the combination of mutations of the LPS synthesis genes and genes of general metabolism has the most severe effect on bacterial fitness, as reported for the clone *Pa_phoQ/lptA/prs*. Further study of the interplay between genotype and phenotype via experimental modeling will improve understanding of the mechanisms underlying adaptation of bacteria to environmental factors, including the development of antibiotic resistance, and outline new ways to overcome bacterial resistance to drugs.

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