MUTATIONAL BASIS OF MEROPENEM RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

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The carbapenem-resistant strains of *Pseudomonas aeruginosa* are considered as the dangerous pathogens of critical priority. Deciphering the mechanisms underlying the development of carbopenem resistance is an urgent challenge faced by modern medical science. The study was aimed to describe the diversity and fixation of mutations associated with the development of carbapenem resistance during the *P. aeruginosa* adaptation to the increasing meropenem concentrations. The objects of the study were *P. aeruginosa* isolates obtained by growing the ATCC 27853 *P. aeruginosa* reference strain exposed to increasing concentrations of meropenem. The isolates were tested for meropenem susceptibility using E-tests (Epsilometer tests) and by the agar dilution method. Genomes of the isolates were sequenced in the MGISEQ-2000 whole-genome sequencer. The findings show that in experimental settings *P. aeruginosa* develops high meropenem resistance very quickly (in 6 days). Evolution of resistance is associated with cloning involving the emergence of multiple clones with various genotypes. Mutagenesis that involves 11 genes, including *oprD*, *pbuE*, *nalD*, *nalC*, *spoT*, *mlaA*, *mexD*, *mexR*, *oprM*, *mraY*, *pbp3*, provides the basis for cloning. Regardless of the levels of their meropenem resistance, some of the emerging clones do not progressively develop and are replaced by more successful clones.

Keywords: antibiotics, resistance, *Pseudomonas aeruginosa*, meropenem, mutation

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Compliance with ethical standards: the study was performed in full compliance with the principles of the Declaration of Helsinki and the standards for handling opportunistic pathogens.

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МУТАЦИОННЫЕ ОСНОВЫ ФОРМИРОВАНИЯ УСТОЙЧИВОСТИ К МЕРОПЕНЕМУ У *PSEUDOMONAS AERUGINOSA*

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Резистентные к карбапенемам штаммы *Pseudomonas aeruginosa* расценивают в качестве критически опасных патогенов первого уровня приоритета. Расшифровка механизмов формирования устойчивости к карбапенемам является актуальной задачей современной медицинской науки. Целью работы было описать разнообразие и закрепление мутаций, ассоциированных с формированием карбапенемрезистентности в процессе адаптации *P. aeruginosa* к повышающимся концентрациям меропенема. Объектами исследования были изоляты *P. aeruginosa*, полученные при росте референтного штамма *P. aeruginosa* ATCC 27853 в градиенте возрастающих концентраций меропенема. Оценку чувствительности изолятов к меропенему выполняли при помощи е-тестов (эпсилометрический метод) с меропенемом и при помощи метода дилюции антибиотика в агаре. Геномы изолятов были секвенированы на полногеномном секвенаторе MGISEQ-2000. Полученные результаты показали, что формирование высоких уровней резистентности к меропенему у *P. aeruginosa* в эксперименте происходит в короткие сроки (6 суток). Эволюция резистентности сопряжена с процессом клонирования, при котором происходит возникновение множества клонов с различными генотипами. Основой клонирования является мутагенез, в который вовлечены 11 генов, включая *oprD*, *pbuE*, *nalD*, *nalC*, *spoT*, *mlaA*, *mexD*, *mexR*, *oprM*, *mraY, pbp3*. Часть образовавшихся клонов, независимо от уровня их резистентности к меропенему, не получают прогрессивного развития и вытесняются более успешными клонами.

Ключевые слова: антибиотики, резистентность, *Pseudomonas aeruginosa*, меропенем, мутации

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Pseudomonas aeruginosa is one of the major opportunistic pathogens [1]. The carbapenem-resistant *P. aeruginosa* strains are especially dangerous for patients, that is why these strains have been included in the WHO priority list for R&D of new antibiotics for antibiotic-resistant bacteria as dangerous pathogens of critical priority [2]. Carbapenem resistance can be developed in two ways. The first way is implemented by acquiring resistance genes from external sources via horizontal transfer. This resistance mechanism that is often referred to as plasmid-borne resistance provides high levels of resistance.

Studying this mechanism is more popular among scientists. Enzymes, the heterogenous β-lactamases of various Ambler classes combined into a group of carbapenemases based on the function, provide the main molecular basis for the horisontally transferred carbapenem resistance. However, there is one more way of developing carbapenem resistance that is not associated with horizontal gene transfer. It is based on the *P. aeruginosa* unique adaptive potential and is implemented through mutational variation in the chromosome genes [3]. Among clinical isolates, *P. aeruginosa* strains isolated from patients with cystic fibrosis are the most vivid examples of mutational antibiotic resistance. Highly resistant strains have been reported, which contain more than 60 genes disrupted by mutations. These genes can be the cause of resistance to various classes of antibiotics [4]. Of those 26 mutant genes can cause carbapenem resistance.

Studying the diversity of mutations that occur during the *P. aeruginosa* adaptation to carbapenems is of interest for prediction of carbapenem resistance evolution among clinical strains. The mechanisms underlying carbapenem resistance are assessed using two methodological approaches: 1) stydying genetic and phenotypic characteristics of the clinical carbapenem-resistant isolates; 2) targeted *in vitro* modeling of carbapenem resistance that involves P. aeruginosa exposure to antibiotic.

The study was aimed to describe the diversity and fixation of mutations associated with the development of carbapenem resistance during the *P. aeruginosa* adaptation to the increasing meropenem concentrations.

The targeted creation of resistant *P. aeruginosa* strains is more often modelled using a series of consecutive transfers of bacteria in the liquid growth media containing the increasing concentrations of antibiotic (from 0 µg/mL to the concentrations that are tens and hundreds of times greater than the minimum inhibitory concentration (MIC)) [5]. We used the other model [6] that was based on evolution of motile bacteria exposed to the increasing antibiotic concentrations. Such an approach makes it possible to isolate the larger number of clones with various genotypes.

METHODS

The ATCC 27853 P. aeruginosa reference strain used as a standard of carbapenem susceptibility (The European Committee on Antimicrobial Susceptibility Testing (EUCAST). EUCAST Clinical Breakpoint Tables v. 12.0. Available at: www.eucast.org) was the object of the study.

The study was carried out using the spatiotemporal model of antibiotic resistance in motile bacteria in accordance with the earlier reported method [7]. We formed five compartments divided by partitions with the depth of 2.0 cm in the 20.0 × 40.0 cm container and filled these compartments with the solid growth medium containing Luria Bertani broth (LB Miller, Becton Dickinson and Co.; USA). The growth medium in the compartments contained sequential concentrations (0, 0,2, 20, 200, 2000 µg/mL) of meropenem (Supelco® Analytical Products, Merck & Co. Inc.; USA). A single layer (about 0.6 cm high) of solid growth medium containing Luria Bertani broth with no meropenem was formed atop of the compartments. It was covered with the layer of semi-solid agar (0.28% of agar) containing Luria Bertani broth with no meropenem. This layer was about 0.8 cm high. The culture of P. aeruginosa was adapted to semi-solid growth medium by the earlier reported method before starting the experiment [7].

Bacterial suspension with optical density equivalent to 0.5 MacFarland standard was used for inoculation. Inoculation was performed by injection into the semi-solid agar to a depth of about 1–2 mm in the A sector (Fig. 1).

Every 24 h, samples were collected from the propagating *P. aeruginosa* growth front and inoculated to Mueller–Hinton agar plates (Becton Dickinson and Co.; USA) in order to gather enough biomaterial for further assessment of phenotypic characteristics (antibiotic resistance profile) and bacterial genome alterations.

Isolates were tested for meropenem susceptibility by determining MICs in two ways: 1) using meropenem E-tests (Epsilometer tests) in accordance with the manufacturer's guidelines (BioMerieux SA; France); 2) using the agar dilution method [8]. The MIC values were not interpreted from a clinical perspective, these were analyzed solely in terms of the MIC dynamics.

Trough meropenem concentrations in the semi-solid agar were assessed 240 h after the start of the experiment by high-performance liquid chromatography (HPLC) using a well-known technique [9].

Bacterial DNA was isolated from the 24-h culture of *P. aeruginosa* isolates grown on Mueller–Hinton agar (Becton Dickinson and Co.; USA) using the QIAamp DNA Mini Kit (Qiagen; Germany) in accordance with the manufacturer's protocol. DNA samples were stored at –20 °C. Ultrasonic fragmentation (Covaris; USA) of bacterial DNA (400 ng) with subsequent end repair and adapter ligation (MGI Tech; China) were used to prepare genomic DNA libraries. DNA libraries were washed with the Agencourt AMPure XP magnetic beads (Beckman Coulter; USA). The concentrations of bacterial DNA and DNA libraries were measured using the Qubit 4 fluorometer (Thermo Fisher Scientific; USA). Whole-genome sequencing was performed using the MGISEQ-2000 platform (MGI Tech; China). The read length was 250 bp. The quality was tested using the FASTQC (Babraham Institute; UK) and Trimmomatic v.0.38 (Usadel Lab; USA) software. Genomes were assembled de novo using the SPAdes 3.14 software [10]. The Contest16S web server was used to control the assembly completeness and eliminate the possibility of contamination. The quality of assemblies was evaluated in QUAST 5.0 [11]. Genomes were annotated using the RAST server [12] and the Prokka software [13].

To detect single nucleotide polymorphisms (SNPs), the short reads were mapped to the reference genome in Snippy [14]. The genome of “null” isolate, i.e. the isolate obtained after the ATCC 27853 *P. aeruginosa* strain adaptation to semi-solid agar that was used to launch the experiment, was used as a reference genome. The SnpEff software was used for annotation of the variants identified and prediction of their effects on the genes [15].

BLASTn tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyze genes in the genomes of all the isolates obtained and amino acid sequences of the gene products. The ResFinder service and AMRFinderPlus algorithm included in the NCBI Pathogen Detection pipeline were used for assessment of resistance determinants [16, 17].

RESULTS

The dynamics of the *P. aeruginosa* propagation across the surface of semi-solid agar towards higher meropenem concentrations is provided in Fig. 1. The edge of the *P. aeruginosa* growth reached the zone with the maximum meropenem concentration in 168 h (7 days), and growth on the entire area of culture medium was observed within 240 h (10 days). At the end of the experiment meropenem concentration in the E sector of semi-solid agar (Fig. 1) was 56 µg/mL.

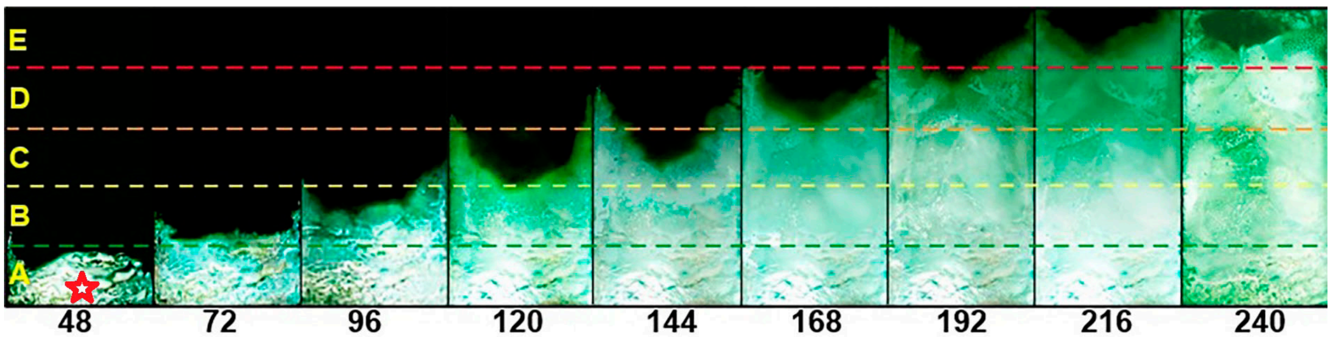

Fig. 1. The dynamics of *P. aeruginosa* propagation across the surface of semi-solid agar towards the higher concentrations of meropenem. The images were acquired after incubation for 48, 72, 96, 120, 144, 168, 192, 216, 240 h from the start of the experiment. The *dashed lines* refer to the boundaries which divide sectors A, B, C, D, E with various meropenem concentrations (0, 0,2, 20, 200, 2000 µg/mL, respectively) in the lower layer of solid growth medium (see Methods). *Asterisk* refers to the starting point (inoculation of the culture of *P. aeruginosa* ATCC 27853)

A total of 92 isolates were collected from the propagating *P. aeruginosa* growth front. Meropenem resistance of the isolates increased as the bacteria propagated towards higher meropenem concentrations (Fig. 2). The increase in MICs from 0.5 µg/mL to 2, 4, and 8 µg/mL was observed within 72 h after the start of the experiment. Isolates with MIC = 16 µg/mL and MIC = 32 µg/mL emerged after 144 h, while isolates with MIC = 64 µg/mL emerged after 216 h. The meropenem MICs > 8 µg/mL were reported in 61 isolates, and MICs ≥ 32 were reported in 45 isolates.

Nonsynonymous mutations were found in 11 genes, including *oprD*, *pbuE*, *nalD*, *nalC*, *spoTм mlaA*, *mexD*, *mexR*, *oprM*, *mraY*, *pbp3*. Mutations of these genes were not detected in four genomes out of 92 (4.3%), these were genomes of isolates obtained in the first 48 h of growth. In other 88 genomes out of 92 (95.7%), various combinations of genes disrupted by mutations were detected (Table 1). The most frequent disrupted genes were *oprD*, *pbuE*, *nalD* (Table 2). Mutations of genes *nalD*, *spoT*, *mlaA*, *mexR*, *mraY*, *pbp3* were associated with high levels of resistance in the isolates carrying these mutations, the meropenem MICs of which exceeded 8 µg/mL (Table 2). In contrast, the *oprM* gene mutations were found only in four strains out of 92 (4.3%) with meropenem MICs exceeding 8 µg/mL. Among 84 strains carrying oprD mutations four highly susceptible isolates with meropenem MICs of 0.5–2 µg/mL were found. In these isolates *oprD* mutations resulted in L292Q, L252P, G307D substitutions in three cases and in premature termination of protein synthesis (W138stop) in one case. The genotype carrying a combination of mutations in *oprD*, *pbuE*, *nalD* was the most common (Table 1).

The dynamics of mutation emergence at various stages of biomaterial collection is provided in Table 2. The first stable mutations emerged in the *oprD* and *pbuE* genes within 72 h after the start of the experiment. The *pbuE* mutation resulting in the A261D substitution was represented by only one variant and was combined with different variants of other mutations evenly in 77 isolates out of 92 (83.7%). The *oprD* mutations were represented by nine variants. However, only two variants of mutations resulting in the G307D (*oprD*-G307D) and L238P (*oprD*-L238P) substitutions were found in the majority of isolates carrying *oprD* mutations (73 out of 84; 86.9%). The other seven variants of *oprD* mutations were relatively rare, these were found in 11 isolates with mutant *oprD* genes out of 84 (13.1%). Thus, the original strain produced two clones, *oprD*-G307D and *oprD*-L238P (Fig. 2). The strain that was a direct ancestor of the clone *oprD*-G307D emerged within 96 h of the experiment and its meropenem MIC was 2 µg/mL. The strain that was a direct ancestor of the clone *oprD*-L238P was not isolated during the experiment. Hypothetically, it could emerge within 120 h after the start of the experiment. Evolution of the main clones, *oprD*-G307D and *oprD*-L238P, was associated with reduction of their meropenem susceptibility (Fig. 2) and accumulation of mutations in other genes important for development of carbapenem resistance.

Starting from hour 144 of the experiment, isolates carrying *nalD* mutation resulting in the G172D substitution emerged among strains of the *oprD*-G307D clone. By the end of the experiment, 14 strains of the *oprD*-G307D clone out of 34 were carriers of this mutation.

The *oprD*-L238P clone was related to the other *nalD* mutations resulting in the T11N (24 isolates of the clone out of 39) and H56P (4 isolates of the clone out of 39) substitutions. The deletion in the *mlaA* gene (5 bp del (nucleotides 423–427)) resulting in the open reading frame shift was also found only in isolates (11 out of 39) of the clone *oprD*-L238P. The *mlaA* deletion was combined with the T11N mutation of the *nalD* gene in all cases.

Mutations of genes *mexR*, *oprM*, *mraY*, *pbp3*, *nalC* were found only in few isolates.

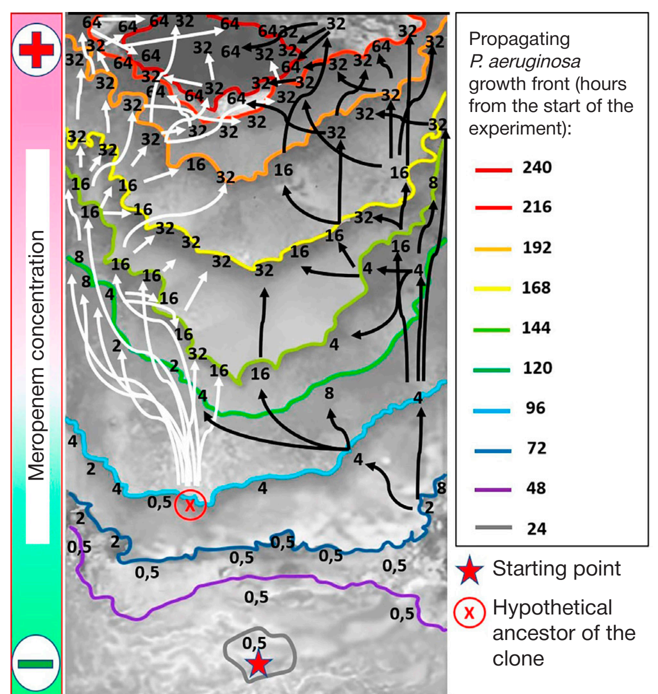

Fig. 2. Topology of *P. aeruginosa* clones on the surface of semi-solid agar with the increasing meropenem concentrations after 240 h of incubation. The numbers refer to meropenem MICs (µg/mL) of isolates collected from the sites designated with the numbers. *White arrows* demonstrate the oprD-L238P clone propagation, *black arrows* demonstrate the oprD-G307D clone propagation

Table 1. Genes and gene combinations where nonsynonymous mutations were found

№	Combinations of genes carrying mutations	Number of strains (% of all strains, *n* = 92)
1	*oprD, pbuE, nalD*	20 (22.2)
2	*oprD, pbuE*	11 (12.0)
3	*oprD, pbuE, nalD, spoT*	10 (10.9)
4	*oprD, pbuE, nalD, mlaA*	11 (12.0)
5	*oprD, pbuE, mexD*	9 (10)
6	*oprD*	6 (7)
7	*oprD, pbuE, spoT*	4 (4)
8	*oprD, pbuE, mexR*	3 (3)
9	*pbuE*	3 (3)
10	*oprD, nalD*	2 (2)
11	*oprD, pbuE, mexR, mraY*	2 (2)
12	*oprD, oprM*	2 (2)
13	*oprM*	1 (1)
14	*oprD, nalC, pbuE*	1 (1)
15	*oprD, pbuE, oprM*	1 (1)
16	*oprD, pbuE, spoT, mexD*	1 (1)
17	*oprD, pbuE, pbp3*	1 (1)
18	No mutations	4 (4)

DISCUSSION

When discussing phenotypic traits of the *P. aeruginosa* adaptation to meropenem, the focus should be placed on the rate of developing resistance. The resistance levels of certain isolates obtained at this stage reached meropenem MICs of 32 µg/mL within 6 days. The maximum meropenem MICs were 64 µg/mL, these were 128 times higher than the MIC values registered in isolates obtained within the first 48 h of the experiment. The fact of finding isolates with MIC values of 32 µg/mL in the zone with the actual meropenem content of 56 µg/mL can be explained by the differences between the conditions of determining MICs by reference methods (Epsilometer test and agar dilution method) and the experimental conditions (growth medium, incubation time).

Gene mutation was revealed along with the meropenem MIC increase in distinct strains on the term of 72 h. A total of 11 mutated genes were found during the experiment. Among those the association with carbapenem resistance was proven only for *oprD*, *nalC*, *nalD*, *mexD*, *mexR*, and *pbp3* [18–21]. The role of *oprM*, *pbuE*, *spoT*, *mraY*, *mlaA* genes in the development of antibiotic resistance has not been reported before, however, this does not eliminate their indirect effects on adaptation to carbapenems.

When considering the mutation pattern as a whole, attention should be paid to the phenomenon of cloning. Two major clonal lines emerged within 72–96 h. All the members of the first clonal line carried the *oprD* mutation resulting in the G307D substitution. The *oprD* mutation resulted in the L238P substitution in all representatives of the other clonal line. New mutations, that resulted in the increased phenotypic resistance to meropenem, emerged and were partially fixed in the clones produced. Along with these lines, single clones carrying other *oprD* mutations emerged. These clones showed no progressive spread, while some of the clones had higher meropenem MICs than the surrounding representatives of the clones *oprD*-G307D and *oprD*-L238P (Fig. 2). Perhaps, mutations in the non-successful but highly resistant clones were the factor adversely affecting the outcome of intraspecific competition. It is worth mentioning that *oprD* disruption in the

Table 2. Meropenem resistant phenotypes of *P. aeruginosa* and genes that can possibly determine carbapenem resistance

№	Gene	Time of mutation emergence (hours since the start)	Number of strains (% of the group) carrying mutations in the groups with various meropenem MICs (µg/mL)			Number of strains carrying mutations (% of all strains, *n* = 92)
			≤ 8, *n* = 31	> 8 < 32, *n* = 16	≥ 32, *n* = 45	
1	*oprD*	72	23 (74,2)	16 (100)	45 (100)	84 (91,3)
2	*pbuE*	72	16 (51,6)	16 (100)	45 (100)	77 (83,7)
3	*nalD*	120	1 (3)	12 (75)	30 (66,7)	43 (46,7)
4	*spoT*	192	0 (0)	1 (6)	14 (31,1)	15 (16,3)
5	*mlaA*	144	0 (0)	1 (6)	10 (22,2)	11 (12,0)
6	*mexD*	120	3 (10)	3 (29)	4 (9)	10 (10,9)
7	*mexR*	144	0 (0)	2 (13)	3 (7)	5 (5)
8	*oprM*	72	4 (13)	0 (0)	0 (0)	4 (4)
9	*mraY*	168	0 (0)	0 (0)	2 (4)	2 (2)
10	*pbp3*	144	0 (0)	0 (0)	1 (2)	1 (1)
11	*nalC*	72	1 (3)	0 (0)	0 (0)	0 (0)

P. aeruginosa meropenem resistant isolates is observed not only in experimental settings. Thus, five highly meropenem resistant (MIC > 32 μg/mL) *P. aeruginosa* strains out of six, which were found in individuals with cystic fibrosis and produced no carbapenemases, carried mutations in the oprD genes [4]. At the same time, disruption of one gene (oprD) is insufficient for development of meropenem resistance. Even the strain carrying the *oprD* nonsense mutation (W138stop termination codon) remained higly susceptible to meropenem. Accumulation of chromosomal mutations in multiple chromosome genes directly or indirectly affecting antibiotic susceptibility is essential for resistance.

We do not exclude the possibility that some isolates with unique genotypes have not been selected during the experiment, and information about these isolates has been lost. The example of this is uncertainty about the progenitor of the *oprD*-L238P clone being an intermediate between the highly susceptible and highly resistant strains. However, in contrast to evolution in liquid medium, spatiotemporal resistance model makes it possible to isolate a larger number of clones and avoid the loss of information about possible mutations leading to resistance.

CONCLUSIONS

In experimental settings *P. aeruginosa* develops high meropenem resistance very quickly (in 6 days). Evolution of resistance is associated with cloning involving the emergence of multiple clones with various genotypes. Mutagenesis that involves 11 genes, including *oprD*, *pbuE*, *nalD*, *nalC*, *spoT*, *mlaA*, *mexD*, *mexR*, *oprM*, *mraY*, *pbp3*, provides the basis for cloning. Regardless of the levels of their meropenem resistance, some of the emerging clones do not progressively develop and are replaced by the more successful clones. The model used during the experiment is a convenient tool to obtain the set of variants with various resistant genotypes.

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