

The Plasmid Differences in Multi-Drug Resistant Opportunistic Pathogenic Soil Strains of *Pseudomonas* and *Stenotrophomonas*

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Abstract

The antibiotic resistance and especially multi-drug resistance is one of the most important factors for any microorganism survival in nature. In a majority of cases the resistance to antibiotics, as a property is being defined by several genes which are localized in plasmids, transposons and in other mobile genetical elements. As a result, it has been found out that in some native opportunistic pathogenic soil strains of *Pseudomonas* and *Stenotrophomonas*, the resistance to different antibiotics is caused by simultaneous presence of different plasmids in cells. Besides, the genes of resistance to various classes of antibiotics of I, II, III generations. They can be localized on one plasmid or in more than one plasmids of current bacterial cell. These plasmids of researched strains of *Pseudomonas* and *Stenotrophomonas* are able to stable replication not only in cells permanently contacting with compatible antibiotic molecules in environment, but also in case of long-term cultivation of bacteria on synthetic media without any antibiotic. The antibiotic resistance of researched *Pseudomonas* and *Stenotrophomonas* strains, which is caused by mobile genetical elements, can be transferred among the microorganisms both in frames of one species and in interspecific and intergeneric gene transfer processes. The plasmids with the presence of genes of resistance to different antibiotics can be transferred to different microorganisms independently, with the forming of new resistant strains, which are differing in resistance to natural antimicrobial organic acids as well as their synthetic derivatives and it has a significant ecological and medical importance.

Keywords: *Pseudomonas*, Plasmids, antibiotics, multi-drug resistance

Introduction

The antibiotic resistance has an invaluable importance for microbe survival in both nature and clinic under the impact of antibiotics and other effects of anthropogenic influence. The genes of resistance can be localized in both nucleoid (bacterial chromosome) and plasmids or other mobile genetic elements [1]. The presence of them increases adaptivity and the survival probability of microbe in terms of changing conditions of environment. In case of plasmid genes of resistance, this property can be transferred among the microbes by the intraspecific horizontal gene transfer, which can result in formation of new antibiotic resistant strains [2, 3]. Different representatives of *Pseudomonas* and *Stenotrophomonas* are characterized by extremely high level of adaptivity and the presence of various systems of resistance to different classes of antibiotics and other toxic natural and synthetic compounds [4, 5]. They include many multi-drug resistant soil strains which are able to be involved in various ecosystems and forming a quorums, phytopathogenic strains, as well as strains which are opportunistic pathogens of human and animals. They differ by the plasmids and their genes content. The plasmids of *Pseudomonas* can carry the genes of antibiotics modification, efflux system and toxic xenobiotics degradation, up to biodegradation of different cyclic and aliphatic hydrocarbons, toluene derivatives and various wastes of oil production [6-11].

The mentioned above properties can be transferred from one bacteria to other during the process of horizontal gene transfer. And it can become a cause of uncontrolled spread of antibiotic resistance from natural non-pathogenic and conditionally pathogenic bacteria to pathogenic microorganisms in clinics. That is why the research of their genetic mechanisms is very actual in ecological and medical aspects. The main aim of this research was a comparison of genetic mechanisms, which are encoding the properties of multi-drug resistance in different native soil strains of *Pseudomonas* and *Stenotrophomonas*. Besides, the researched strains and obtained transformants were compared by their resistance to newly synthesized derivatives of tartaric acid, which were elaborated and tested in our laboratory, based on literature data about antimicrobial activity of tartaric acid and the derivatives of it [12-14].

Materials and Methods

Cultivation of Cultures

In current research there were used the strains from The National Culture Collection of Microorganisms of the Microbial Depository Center of "Armibiotechnology" Scientific and Production Center National Academy of Sciences, Republic of Armenia: *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* (*S. maltophilia* or former *P. maltophilia*), *P. chlororaphis* (subsp. *Chlororaphis*, subsp. *Aurantiaea*, subsp. *Aureofaciens*), *P. taetrolens*, *P. putida*, *P. fluorescens* and *P. geniculata*. There were cultivated on liquid and solid agarised media at 37°C [15].

Antibiotic resistance test

The resistance of all strains was tested by cultivation on agarised cultural media, containing 50mg/ml compatible antibiotic. There were used antibiotics of different classes and different generations: β -lactamic (Amp/ampicillin, Amx/Amoxicillin, Amc/Augmentin, Cfx/Cefixime and Ctx/Ceftriaxone); aminoglycoside (Kan/kanamycin, Stp/Streptomycin); fluoroquinolone (Cip/Ciprofloxacin); Tcn/Tetracycline, Macrolides (Azm/azithromycin); amphenicol (Cam/Chloramphenicol) produced by "Astoria" [16, 17]. As the control strains there were used *E. coli* DH5 α (sensitive to all mentioned above antibiotics), *E. coli* DH5 α /pUC18 (resistant to Ampicillin) and *E. coli* DH5 α /VOG 16 (resistant to kanamycin). Antimicrobial activity of Tartaric acid, benzylimide and cyclohexyl amide derivatives was tested according the standard protocols in concentration 1-6% [18].

Analysis of bacterial DNA

The isolation of plasmid and total DNA was carried out by alkaline extraction method and by the method with the use of benzyl chloride. Then the isolated DNA was researched by 0.8-2.5% agarose gel electrophoresis [19,20]. The transformation of sensitive strains by the plasmid DNA of resistant strains was realized by Mandel's method of competent cells with CaCl₂ usage [21-24]. PCR analysis of DNA from the cells of all donor, recipient and transformant strains was done with the following primers: *aph(3')IV*, *bla_{OXA-10}*, *aac(6')II*, *pCAT639*: As the marker it was used the standard mix of DNA fragments EcoRI/Hind III [25-28].

Results

The resistance of more than 70 soil strains of 7 species of *Pseudomonas* and *Stenotrophomonas* to: β -lactamic antibiotics of different generations (Amp, Amx, Amc, Cfx, Ctx); amphenicols (Cam) and aminoglycosides (Stp, Kan) was researched. As a result, the strains which are mono-, multi-drug resistant and sensitive to antibiotics were detected. One part of them was resistant not only to β -lactamic antibiotics, but also the growth of them couldn't be inhibited by clavulanic acid of augmentin. Then the DNA samples were isolated from all strains and the plasmid content of their cells was compared.

The identification of resistance genes was done by PCR analysis. For the definition of resistance genes localization, the sensitive strains *P. aeruginosa* 9056 and *E. coli* DH5 α were transformed by the plasmids from the resistant strains and then the transformed strains were selected on different selective media with compatible antibiotics (Table 1).

During the experiments it was found out that for 4 strains, the resistance of transformants differed from the donor's resistance. The resistance of *P. aeruginosa* 9056 and *E. coli* DH5 α transformed by the same plasmids was identical. The correlation between transformation and PCR analysis data are presented in Table 2.

The results of antimicrobial effect differences while tests with derivatives of tartaric acid are shown on Table 3.

Discussion

As it was shown above, in resistant strain *P. aeruginosa* 9059 two different plasmids were detected. In one of them acetyl-Co-A-dependent aminoglycoside N-acetyltransferase gene *aac* (6') II, which defined the resistance to Kan, was identified.

The gene of chloramphenicol acetyltransferase CatB7 *pCAT639* is identified in bacterial chromosome and that is why this property cannot be transferred, just as the resistance to Stp, which is encoded by chromosomal genes too. The second plasmid of this strain contains the genes of resistance to β -lactamic antibiotics. Both plasmids can transfer the resistance to other microorganisms by intraspecific horizontal gene transfer [29]. According to the collected data, the cells which were transformed by the plasmids of multi-drug resistant strain *P. aeruginosa* 5249, containing *bla*OXA10 β -lactamase gene *bla*_{OXA10}, *pCAT639* and ATP-dependent aminoglycoside O-phosphotransferase gene *aph* (3') VI, were sensitive to Stp and Cam. It is caused by the plasmid localization of genes *bla*_{OXA10} and *aph* (3') VI and chromosomal localization of *pCAT639* [30, 31].

In *S. maltophilia* 306d2 – there were detected 2 types of plasmids, which can be transferred separately. In one of them the gene *bla*OXA10, while in the second one gene *aph* (3') VI are identified. Probably, the resistance to both aminoglycosides and β -lactams, which was detected for one type of transformants, containing this plasmid, is caused by the genes of another lactamase or efflux system [32]. In *S. maltophilia* 9289 the genes *bla*_{OXA10} and *aac* (6') II were detected on different plasmids which could be transferred independently, while the resistance to Cam was defined by chromosomal genes. According to data from table 3, the transformants, which were selected on aminoglycosides are more resistant. Thus probably, the plasmids of these strains carry additional genes of degradation or efflux of tartaric acid and the derivatives of it, while the plasmids with genes of resistance to β -lactamic antibiotics have no relation to this property.

Conclusion

As the result of experiments among the researched multi-drug resistant microbes, the strains with different plasmids and more than one plasmid simultaneously were detected. These plasmids carry different genes of resistance to β -lactams and aminoglycosides and are able to stable replication even after the long-term cultivation on media without antibiotics. The resistance to chloramphenicol in all these strains is caused by chromosomal genes. In some cases, the resistance to β -lactams is caused by both chromosomal and plasmid genes. For 2 strains of *P. aeruginosa* and 2 strains of *S. maltophilia* an ability to transfer the resistance to aminoglycosides and to β -lactams independently by 2 different plasmids with additional genes of resistance to natural aldaric acids like tartaric acid, by the intraspecific horizontal gene transfer, was

shown and it has a huge ecological significance for new antibiotic resistant strains formation and spread in nature.

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Table 1. Sensitive strains transformation by plasmid DNA of resistant strains.

(R- resistance; S- sensitivity; + the growth on media; - the absence of growth).

Donor and the antibiotic of election	Donor's Resistance	Resistance of transformants							
		Amp	Amx	Amc	Cfx	Ctx	Kan	Stp	Cam
<i>P. aeruginosa</i> 5249 (on β -lactams, Cam & on aminoglycosides)	R, Azm ^S	+	+	+	+	+	+	-	-
<i>P. aeruginosa</i> 9059 (on β -lactams, Cam)	R, Stp ^S , Ctx ^S	+	+	+	+	-	-	-	-
<i>P. aeruginosa</i> 9059(on Kan)		-	-	-	-	-	+	-	-
<i>S. maltophilia</i> 306d2(on β -lactams, Cam)	R, Cam ^S	+	+	+	-	-	-	-	-
<i>S. maltophilia</i> 306d2 (on aminoglycosides)		+	+	+	-	-	+	-	-
<i>S. maltophilia</i> 9289 (on β -lactams, Cam)	R	+	+	+	-	-	-	-	-
<i>S. maltophilia</i> 9289 (on aminoglycosides)		-	-	-	-	-	+	-	-

Table 2. Correlation between PCR analysis and plasmid consistence of cells.

(R- resistance; S- sensitivity; + the growth on media; - the absence of growth).

Pseudomonas	Resistance	Plasmids	PCR
<i>P. aeruginosa</i> 9059	R, Ctx ^S	2 plasmids	aac (6') II - 2,2kb pCAT639 - 1,4kb
<i>P. aeruginosa</i> 5249	R, Azm ^S	1 plasmid	bla _{OXA} 10-1,6kDa aph (3') VI- 2kDa pCAT639-1,4kDa
<i>S. maltophilia</i> 306d2	R, Cam ^S	2 plasmids	bla _{OXA} 10 - 1,6kb aph(3')VI- 2kDa
<i>S. maltophilia</i> 9289	R	2 plasmids	bla _{OXA} 10 - 1,6kb aac (6') II - 2,2kb

Table 3. Antimicrobial effect of tartaric acid and the derivatives of it on obtained transformant strains of *Pseudomonas* and *Stenotrophomonas*, which were selected on different antibiotics.

(Na₂-TA – Disodium salt of L- tartaric acid, Na/K-TA – Sodium potassium L(+)-tartrate tetrahydrate, TA – L-Tartaric Acid, CHA – NH₄⁺-salt of Cyclohexyl amide of L-tartaric Acid, BATA – NH₄⁺-salt of Benzilimide of L-Tartaric Acid, C – Control sample on Agarised cultural media, + the growth of bacteria on media; - the absence of growth of bacteria on media).

Strain of bacteria	Testing Antimicrobial compound					
	Na ₂ -TA	Na/K-TA	TA	BATA	CHATA	C
<i>P. aeruginosa</i> 5249 (on β-lactams, Cam & on aminoglycosides)	+	+	+	-	-	+
<i>P. aeruginosa</i> 9059 (on β-lactams, Cam)	+	+	+	-	+	+
<i>P. aeruginosa</i> 9059 (on Kan)	-	+	-	+	-	+
<i>S. maltophilia</i> 306d2 (on β-lactams, Cam)	-	-	+	-	-	+
<i>S. maltophilia</i> 306d2 (on aminoglycosides)	-	-	+	-	-	+
<i>S. maltophilia</i> 9289 (on β-lactams, Cam)	+	+	+	-	-	+
<i>S. maltophilia</i> 9289 (on aminoglycosides)	+	+	+	+	+	+