ISSN 2601-6397 (Print)
ISSN 2601-6400 (Online)
European Journal of Medicine and Natural Sciences
January - April 2019
Volume 3, Issue 1

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

Bella Babayan

Scientific & Production Center "Armbiotechnology", National Academy of Sciences, Republic of Armenia,

National Polytechnic University of Armenia, faculty of Chemical Technologies and Environmental Engineering

Abstract

The antibiotic resistance and especially mult-idrug 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 stabile 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 acidcs 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, phytopahogenic 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 medial 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 transtformants 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 "Armbiotechnology" 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. Aurantiaca, subsp. Aureofaciens), P. taetrolens, P. putida, P. fluorescens and P. geniculata*. There were cultivated on liquid and solid agarised media at 37˚C [15].

Atibiotic resistance test

The resistance of all strains was tested by cultivation on agarised cultural media, cotaing 50mkg/ml compatible antibiotic. There were used antibiotics of different classes ad 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 DH5α* (sensitive to all mentioned above antibiotics), *E. coli DH5α*/pUC18 (resistant to Ampiciline) and *E. coli DH5α*/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].

Analysys of bacterial DNA

The isolation of plasmid and total DNA was carried out by aaalkalie extraxtion 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 $CaCl_2$ 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 resisrant not only to β-lactamic antibiotics, but also the growth of them couldn't be inhibited by to 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 DH5α* 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 DH5α* 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 stain *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 blaOXA10 β-lactamase gene *bla$_{OXA}$10*, *pCAT639* and ATP-dependent aminoglycoside O-phosphotransferase gene *aph (3') VI,* were sensitive to Stp and Cam. It is caused by the plasmid localization of genes *bla$_{OXA}$10* 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 *blaOXA10,* 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$_{OXA}$10* and *aac (6') II* were detected on different plasmids which could be transferred independently, while the resistance to Cam was defined by chromosomal genes. Accordig to data from table 3, the transformants, which were selected on aminoglycosides are more resistant. Thus probably, thy plasmids of these strains carry additional genes of degradation or efflux of tartaric acid and the derivatives of it, whike 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.

This work was supported by the RA MES State Committee of Science, in the frames of the research project № 18T-2I036.

References

[1] Li E. Zh., Dawei Zh. D. X., Jin X., Chunguang Y., Hao F., Zhouhua J., Li X., Tingyue G., Ke Y. (2016, February) Microbiologically Influenced Corrosion of 2707 Hyper-Duplex Stainless Steel by Marine *Pseudomonas aeruginosa* Biofilm Huabing, Sci Rep., 6 (20190).
[2] Felker P, Medina D, Soulier C, Velicce G, Velarde M, Gonzalez C. (2005, April) A survey of environmental and biological factors (*Azospirillum spp., Agrobacterium rhizogenes*, *P. aurantiaca*) for their influence in rooting cuttings of Prosopis alba clones. J Arid Environ. 61 (2): 227–247.
[3] Dashchyan N.A., Asatryan N.L., Galstyan G.F., Mikaelyan A.R. (2014, November), Obtaining Bioactive Additives of Cyclic Structure on the Basis of Optically Active Tartaric Acid. Bulletin of NPUA, Collection of scientific papers, part II, pp. 682-68CLSI,
[4] Gharajyan S.K, Babayan B.G., Sogomonyan T.M., Mikaelyan A. R, Melkumyan M.A., Baghdasaryan A. S. (2018, November), The Influence of Tartaric Acid and The Derivatives of It On Some Soil Non Pathogenic Strains of Pseudomonas, The materials of Conference: "The Assessment of Biodiversity and Agro-Biodiversity Capacity of the RAand the Implementation of the Scientific-Educational Foundation for Biodiversity Conservation", ASPU, 1(1), 171-174
[5] Performance Standards for Antimicrobial Susceptibility Testing (2017, January), Suplement M100S, 27th ed.
[6] CMI, ESCMID, ECAST, Terminology relating to methods for the determination of susceptibilityof bacteria to antimicrobial agents (2010, May), ESCMID 6, 4503-508.
[7] Lucotte G., Baneyx F. (1993). Introduction to Molecular Cloning Techniques. Wiley-Blackwell. p. 32. ISBN 978-0471188490.
[8] Blanco P., Hernando-Amado S., Reales-Calderon J.A., Do Corona F., Lira F., Alcalde-Rico M., Bernardini A., Sanchez M. B., Martinez J. L. (2016 March) Bacterial Multi-drug Efflux Pumps: Much More Than Antibiotic Resistance Determinants, Microorganisms. 4(1): 14.
[9] Maurya A. P., Dhar D., Basumatary M. K., Paul D., Ingti B., Choudhury D., Talukdar A. D., Chakravarty A., Mishra S., Bhattacharjee A. (2017,April) Expansion of highly stable blaOXA-10 β-lactamase family within diverse

host range among nosocomial isolates of Gram-negative bacilli within a tertiary referral hospital of Northeast India", BMC Res Notes. 10(1):145..

[10] Xiumei H., Banglao X., Yinmei Y., Liu D., Yang M, Wang J., Shen H., Zhou X., Ma X. (2013, March) A high throughput multiplex PCR assay for simultaneous detection of seven aminoglycosideresistance genes in *Enterobacteriaceae*, Hu et al. BMC Microbiology, 13, 58.

[11] Jovcić B, Lepsanović Z, Begović J, Rakonjac B, Perovanović J, Topisirović L, Kojić M. (2013, July)The clinical isolate *Pseudomonas aeruginosa* MMA83 carries two copies of the blaNDM-1 gene in a novel genetic context." Antimicrob Agents Chemother, 57(7):3405-7.

[12] Tanner W.D, Atkinson RM, Goel R.K., Toleman M.A, Benson LS, Porucznik CA, VanDerslice JA. (2017, April) Horizontal transfer of the *blaNDM-1* gene to *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in biofilms." FEMS Microbiol Lett, 364(8).

[13] Rampioni G., Pillai C. R., Longo F., Bondì R., Baldelli V., Messina M., Imperi F., Visca P., Leoni L. (2017, September) Effect of efflux pump inhibition on *Pseudomonas aeruginosa* transcriptome and virulence, Sci Rep., 7: 11392

[14] Heng Zhu, Feng Qu, Li-Huang Zhu. (1993, November) Isolation of Genomic DNA From Plants, Fungi and Bacteria Using Benzyl Chloride. Nucleic Acid Research, 21 (22), 5279-5280.

[15] Viovy J.-L. (2000, July). Electrophoresis of DNA and other polyelectrolytes: Physical mechanisms. Reviews of Modern Physics. 72: 813–872.

[16] S. Eswaranandam N. S. Hettiarachchy M. G. Johnson (2004, April), Antimicrobial Activity of Citric, Lactic, Malic, or Tartaric Acids and Nisin-incorporated Soy Protein Film Against Listeria monocytogenes, Escherichia coli O157:H7, and Salmonella gaminara, Journal of Food Science, 69(3), FMS79-FMS84

[17] Wang J, Liu J.-H. (2004 June) Mutations in the chloramphenicol acetyltransferase (S61G, Y105C) increase accumulated amounts and resistance in *Pseudomonas aeruginosa*, School of Life Science & Technology, Shanghai Jiaotong University, No. 800, China Received, 197-204.

[18] Madigan M, Martinko J. (2005, January) Brock Biology of Microorganisms. (11th ed.). Prentice Hall. ISBN 0-13-144329-1.

[19] Haas D, Défago G. (2005, May) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol. 3(4):307-19.

[20] Shen JP, Zhang LM, Zhu YG, Zhang JB, He JZ. (2008, June) Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam., Environ Microbiol., 10(6):1601-11.

[21] Kaiser S., Biehler K., Jonas D. (2009, May) A Stenotrophomonas maltophilia Multilocus Sequence Typing Scheme for Inferring Population Structure, J Bacteriol., 191(9): 2934–2943.

[22] Singh M., Yadav A., Ma X., Amoah E. (2010, April) Plasmid DNA Transformation in *Escherichia Coli*: Effect of Heat Shock Temperature, Duration and Cold Incubation of CaCl 2 Treated Cells, International Journal of Biotechnology and Biochemistry, 6 (4), 561–568

[23] Marcelletti S, Scortichini M. (2014, December) Definition of Plant-Pathogenic *Pseudomonas genomospecies* of the *Pseudomonas syringae* Complex Through Multiple Comparative Approaches, Phytopathology, 104(12): 1274-82.

[24] Lee J., Zhang L. (2015, January), The hierarchy quorum sensing network in *Pseudomonas aeruginosa*, Protein Cell, 6(1): 26–41.

[25] S.S. Hoseini and M. G. Saue (2015, January) Molecular cloning using polymerase chain reaction, an educational guide for cellular engineering, J. Biol Eng., 9, 2.

[26] Silva M.M., Lidon F.C (2016, January), An overview on applications and side effects of antioxidant food additives, Emirates Journal of Food and Agriculture, 28(12): 823-832

[27] Lateef B. Salam (2016, June) Metabolism of waste engine oil by *Pseudomonas* species. 6 (1) doi: 10.1007/s13205-016-0419-5.

[28] Liu Y, Wang H, Cui T, Zhou X, Jia Y, Zhang H, He ZG (2016, July) NapM, a new nucleoid-associated protein, broadly regulates gene expression and affects mycobacterial resistance to anti-tuberculosis drugs.", Mol Microbiol.,101(1):167-81.

[29] Rohde A., Hammerl J.A., and Dahouk S. A. (2016, September). Rapid screening for antibiotic resistance elements on the RNA transcript, protein and enzymatic activity level, Ann Clin Microbiol Antimicrob, 5 (55), 2-8, DOI 10.1186/s12941-016-0167-8.
[30] Baumrin E, Piette E.W., Micheletti R.G (2017, December) *Stenotrophomonas maltophilia*: an emerging multi-drug resistant opportunistic pathogen in the immunocompromised host., BMJ Case Rep. pii: bcr-2017-221053.
[31] Subedi D, Vijay A.K., Willcox M. (2018, March) Overview of mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*: an ocular perspective, Clin Exp Optom.; 101(2):162-171,
[32] S. Delaney, R. Murphy, F. Walsh (2018, August) A Comparison of Methods for the Extraction of Plasmids Capable of Conferring Antibiotic Resistance in a Human Pathogen From Complex Broiler Cecal Samples, Front Microbiol. 9: 1731
[33]

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 Resis-tance	Resistance of transformants							
		Amp	Amx	Amc	Cfx	Ctx	Kan	Stp	Cam
P. aeruginosa 5249 (on β-lactams, Cam & on aminoglycosides)	R, Azm^S	+	+	+	+	+	+	-	-
P. aeruginosa 9059 (on β-lactams, Cam)	R, Stp^S, Ctx^S	+	+	+	+	-	-	-	-
P. aeruginosa 9059(on Kan)		-	-	-	-	-	+	-	-
S. maltophilia 306d2(on β-lactams, Cam)	R, Cam^S	+	+	+	-	-	-	-	-
S. maltophilia 306d2 (on aminoglycosides)		+	+	+	-	-	+	-	-
S. maltophilia 9289 (on β-lactams, Cam)	R	+	+	+	-	-	-	-	-
S. maltophilia 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
P. aeruginosa 9059	R, Ctx^S	2 plasmids	aac (6') II – 2,2kb pCAT639 - 1,4kb
P. aeruginosa 5249	R, Azm^S	1 plasmid	bla_{OXA}10-1,6kDa aph (3') VI– 2kDa pCAT639-*1,4kDa*
S. maltophilia 306d2	R, Cam^S	2 plasmids	*bla_{OXA}10* - 1,6kb aph(3')VI– 2kDa
S. maltophilia 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.

(Na_2-TA – Disodium salt of L- tartaric acid, Na/K-TA – Sodium potassium L(+)-tartrate tetrahydrate, TA – L-Tartaric Acid, CHA – NH_4^+-salt of Cyclohexyl amide of L-tartaric Acid, BATA – NH_4^+-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
P. aeruginosa 5249 (on β-lactams, Cam & on aminoglycosides)	+	+	+	-	-	+
P. aeruginosa 9059 (on β-lactams, Cam)	+	+	+	-	+	+
P. aeruginosa 9059 (on Kan)	-	+	-	+	-	+
S. maltophilia 306d2 (on β-lactams, Cam)	-	-	+	-	-	+
S. maltophilia 306d2 (on aminoglycosides)	-	-	+	-	-	+
S. maltophilia 9289 (on β-lactams, Cam)	+	+	+	-	-	+
S. maltophilia 9289 (on aminoglycosides)	+	+	+	+	+	+