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Characteristics of *Serratia Marcescens* Strains

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Abstract: One of the most topical issues in the modern world is increase in antibiotic resistance of bacteria. Progressing threat of development of antimicrobial resistance is related to the use of antibiotics for treatment of humans and animals as well as by manufacturing of food products. The annually increasing number of fatal cases is related to the absence of mew antibiotics as well as to great financial costs required for the procedure of implementation thereof in the medical practice. Representatives of Serratia genus are able to cause wide range of infectious diseases. Treatment of such diseases is often hindered because of the multiple antibiotic resistances of bacteria. Within the study we performed identification of phylogenetic affinity of the chromogenic (SM6) and pigment-free (SR41-8000) strains of *Serratia Marcescens* on the basis of comparison of nucleotide sequences of fragments of the 16S rRNA gene. It was found that the SM6 and SR41-8000 strains are located in different clusters of the phylogenetic tree. Proteolytic and nuclease activity of strains as well as sensitivity to antimicrobial agents of two *S. Marcescens* strains were compared. Nuclease activity in the cell culture fluid of *S. Marcescens* SR41-8000 exceeded nuclease activity of the *S. Marcescens* SM6 strain whereas the extracellular proteolytic activity was higher in the *S. Marcescens* SM6 strain. The *S. Marcescens* SM6 strain featured higher resistance to different classes of antibiotics than the *S. Marcescens* SR41-8000 strain.

Key words: *Serratia Marcescens*, protease, nuclease, antibiotic resistance, SM6 strain

INTRODUCTION

Today, the issue of antimicrobial resistance is especially acute. During the last decades, the pathogens causing common infections developed resistance to new antibiotics which has become the global-scale problem. (WHO, 2013). Thus, the necessity of understanding of molecular mechanisms of origination of bacterial antibiotic resistance is obvious.

Many diseases of humans and animals are caused by bacteria of the Enterobacteriaceae family. In contrast to these pathogenic microbes, the *Serratia Marcescens* bacterium usually resides outside the human body. Due to this, it has been thought for a long time that *S. Marcescens* is not a pathogen and only during the last years the data was obtained that *S. Marcescens* is one of the dangerous agents causing hospital-acquired infections (Abbott, 1999; Hejazi, 1997; Mahlen, 2011; Ostrowsky, 2002).

Despite, the numerous descriptions of infections caused by *S. Marcescens*, the mechanisms of virulence of this organism are still underinvestigated. It is known that *S. Marcescens* secrets many known extracellular proteins including chitinases, lecitinases, hemolysins, lipases, proteases and nucleases (Hines, 1988). Since, virulence is individual for any strain the objective of this study was determination of the degree of kinship of different isolate strains *S. marcencens* (chromogenic and pigment-free) and well as comparative characteristic thereof by extracellular hydrolytic activity and antibiotic sensitivity.

MATERIALS AND METHODS

Bacteria strains used: In the study, two isolate strains Serratia Marcescens *S. Marcescens* SM6 and *S. Marcescens* SR41-8000 were used that were kindly furnished by Michael Benedict (USA). By cultivation the *S. Marcescens* SM6 strain releases a red pigment, the *S. Marcescens* SR41-8000 strain is a pigment-free strain.

Estimation of proteolytic activity: In order to estimate the protease activity azo-casein was used as substrate according to the method (Sabirova *et al*., 2010). Measurements were performed with the use of spectro

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photo meter (Bio-Rad, USA) at the wavelength 450 nm. The amount of enzyme hydrolyzing under the experimental conditions 1 μg of substrate per 1 mL of enzyme solution per 1 min was taken as the unit of activity. The culture capacity (specific activity) was determined as ratio of the enzyme activity in the cell culture fluid to the biomass amount and was expressed in conventional units (cu) or in percentage.

Inhibitors impact of proteinase activity: In order to determine the inhibitors impact on activity of proteinases, enzyme was kept in the presence of reagent for 1 h at room temperature after which the residual activity was estimated by azo-casein hydrolysis. The residual activity was expressed as percentage as compared to the control group the activity of which was taken to be equal to 100% in the absence of inhibitors in the reaction mixture. In the study we used: specific inhibitor of serine proteinases PMSF, inhibitor of metalloproteinase 1,10-phenanthroline. Inhibitors were added in the enzyme solution at final concentration of 5 mM.

Estimation of nuclease activity: Activity of ribonuclease in the cell culture fluid of bacteria was estimated by the RNA hydrolysis products dissolved in 4% $HClO_4$ with 12% of uranyl acetate. The enzyme amount causing increase in E_{260} by 1 opt. unit equivalent to 1 ml of enzyme solution per 1 h of incubation was taken as the unit of activity. Screening of RNA activity in strains was performed on plates with test medium containing RNA solution (2 mg mL) and toluidine blue stain (0.001%) (Leschinskaya *et al*., 1974).

Determination of antibiotic resistance using the disk diffusion method: In the disk diffusion method a paper disk is used as the AID (anti-infective drug) carrier. Formation of the area of inhibition of growth of microorganisms inoculated on the agar surface proceeds as the result of AID diffusion from the carrier into the nutritional medium. Within certain limits the value of the growth inhibition area diameter is inversely proportional to the Minimal Inhibitory Concentration of antibiotic (MIC) (Anonymous, 1890). To estimate the DDM sensitivity the agarized (2%) nutritional medium LB was used. To estimate the DDM sensitivity the commercial standardized disks were used (produced by St. Petersburg Pasteur Institute, St. Petersburg).

DNA purification: DNA from the selected strains was isolated using the Fermentas Genomic DNA Purification Kit (Thermo Scientific, Lithuania). The 16S rRNA genes of selected isolates were amplified using Polymerase Chain Reaction (PCR) by standard 27F forward primer (5' GAGTTTGATCCTGGCTCAG 3') and 1492R reverse primer (5' TACCTTGTTACGACTT 3'). The PCR-products were purified using the Fermentas PCR purification kit (Thermo Scientific, Lithuania) and sequenced by the Interdisciplinary Center for Collective Use of Kazan Federal University, Russia. The DNA sequences were compared with the sequences in GenBank database using the BLAST internet tool.

Phylogenetic analysis: It was performed with the use of the MEGA (Version 6.06) software. Nucleotide sequences of 16S rRNA genes were taken from the NCBI database. The phylogenetic tree was designed using the border combination method with the use of the pairwise deletion option. Validity of the tress obtained was controlled with the use of parameters of initial download of 500 repeats and P-distances of amino acids.

RESULTS AND DISCUSSION

Design of phylogenetic tree: The common phylogenetic marker for bacteria is sequencing of the 16S gene of ribosomal RNA (16S rRNA) that allows by comparing sequences of the 16S rRNA gene with the known sequences from the database referring the bacteria kinds being investigated to different phylum. The 16S rRNA gene has highly conversed and variable regions the analysis of which provides essential evolution information (Amann, 1995). In order to specify the taxonomic position of the *S. Marcescens* SM6 and *S. Marcescens* SR41-8000 strains we have performed genetic analysis. The ribosomal RNA gene was amplified using PCR with formation of the fragment≈1500 base pairs long.

For the purpose of identification of phylogenetic kinship on the basis of comparison of nucleotide sequences of the 16S rRNA gene fragment we obtained the sequences of the relevant genome fragments. Using the software application MEGA (Version 6.06) they were compared to the sequences from the BLAST database. As the out-group we've chosen representatives of Escherichia and Yersinia genus. The phylogenetic tree is presented in Fig. 1.

According to the results obtained, the *S. Marcescens* SM6 and *S. Marcescens* Db11 strains are united into a single cluster and the *S. Marcescens* SR41-8000 strain belongs to another cluster. Thus, the *S. Marcescens* SM 6 strain, according to analysis of the sequence of the 16S rRNA gene is closely related to the strain *S. Marcescens* Db 11 causing insect diseases as well as to *S. Marcescens* S52, *S. Marcescens* PiHa5II and *S. Marcescens* CPO1(4) CU strains extracted from the plant rhizosphere (Flyg, 1980; Kamoua, 2015; Rascoe, 2003). Another strain investigated by us,

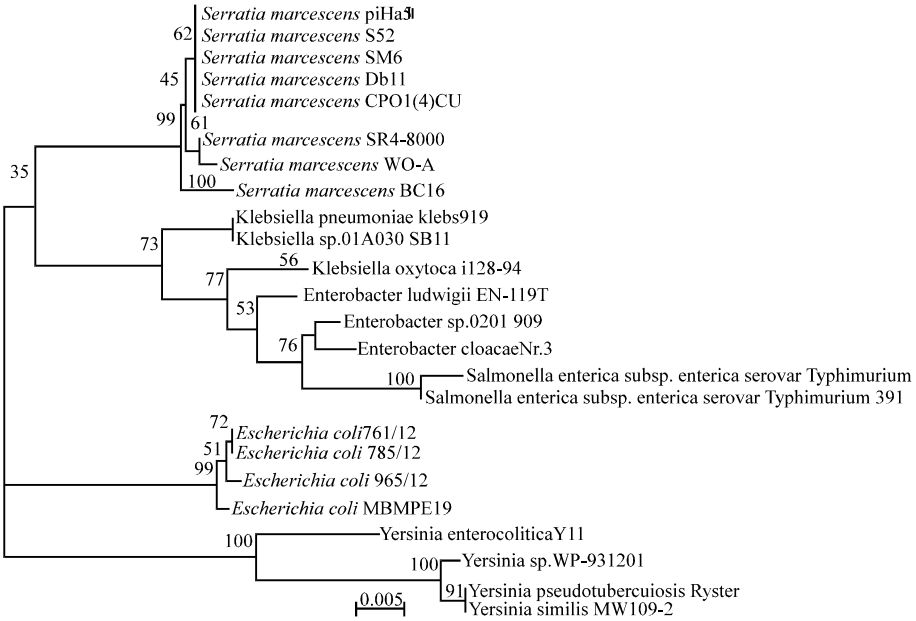

Fig. 1: Phylogenetic trees designed on the basis of structural analysis of the 16S rRNA gene fragment

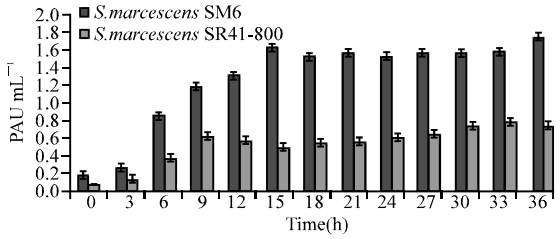

Fig. 2: Extracellular proteolytic activity of *S. Marcescens* strains

S. marcescens SR41-8000 is evolutionary located in another cluster far removed from the group and is phylogenetically close to the *S. Marcescens* WO1-A strain causing gourds diseases (Rascoe, 2003; Bruton, 2003).

For the purpose of further comparison of the *S. Marcescens* strains we analyzed hydrolytic activities (proteolytic and nuclease) of these strains and their resistance to anti-microbial drugs.

Comparison of extracellular hydrolytic activity: Proteolytic proteins hold the key position among the enzymes used in practice. Proteases represent a complex group of enzymes catalyzing splitting of peptide bonds (Francisco, 2008). The strain ability to release proteolytic enzymes in the cultivation medium was investigated.

Our data showed that the maximum proteolytic activity was observed in the cell culture fluid of *S. Marcescens* SM6 (Fig. 2). Protease was found in the medium by the 3d h of cultivation, its activity reached the maximum level by the 15th h and was maintained at high level for 36 h of cultivation. The *S. marcescens* SR41-8000 strain was characterized by twice as small extracellular proteolytic activity than the activity of the *S. Marcescens* SM6 strain.

S. Marcescens bacteria are described by proteinase producers. When *S. Marcescens* is cultivated on protein substrates they produce two kinds of extracellular proteinases: dominant metalloproteinase that is widely used as an anti-inflammatory agent and minor serine proteinase that is described as subtilisin homologue (Rao, 1998). In order to identify belonging of the extracellular strain proteinase to one of the specified classes the impact of different inhibitor on protease activity was studied. It was established that proteolytic enzyme in the cell culture fluid of both strains is not inhibited by PMSF (concentrations 1.1 U mL^{-1} in *S. Marcescens* SM6 and 0.42 U mL^{-1} in *S. Marcescens* SR41-8000) but nearly completely inhibited by 1,10-phenanthroline (concentrations 0.09 U mL^{-1} in *S. Marcescens* SM 6 and 0.1 U mL^{-1} in *S. marcescens* SR41-8000). This data meant that the enzyme of strains being investigated referred to the metalloproteinase class (Fig. 3). In case of simultaneous use of both inhibitors the activity was reduced. Apparently, serine proteinase was present in the cell culture fluid of strains under investigation, yet, on a limited scale.

Along with protease, the ability of *S. Marcescens* strains to release extracellular nuclease was investigated.

Table 1: Sensitivity of *Serratia Marcescens* strains to antibacterial drugs

The class of antibacterial drug	Antibacterial drug	The contents in the disk (μg)	The diameter of the growth inhibition zone, mm (±0.5mm) *S. Marcescens*	
			SM6	SR41-8000
Aminoglycosides II generation	Gentamicin	10	20	27
Aminoglycosides III generation	Amikacin	30	19	27
Cephalosporins I generation	Cefazolin	30	0	15
Cephalosporins III generation	Ceftazidime	30	29	29
	Cefotaxime	30	28	31
Fluoroquinolones I generation	Ciprofloxacin	5	32	37
Hydroxyquinoline	Nitroxoline	20	14	20
Macrolides	Azithromycin	15	15	20
Nitrofurans	Nitrofurantoin	300	13	12
Quinolones	Nalidixic acid	30	32	37
Semisynthetic penicillins	Ampicillin	10	0	25
Tetracyclines	Doxycycline	30	17	17

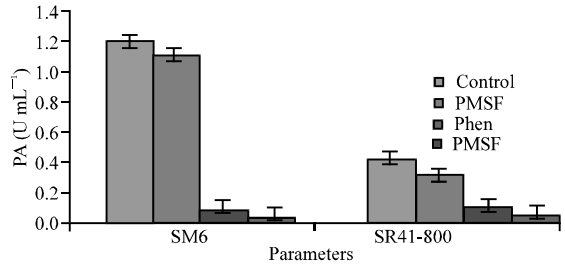

Fig. 3: Impact of inhibitors on proteolytic activity of the cell culture fluid of wild strains *S. Marcescens* SM6 and SR41-8000. Phen (1, 10-phenanthroline) metallo proteinase inhibitor, PMSF (phenyl-methane-sulfonyl-fluoride) inhibitor of serine proteinases

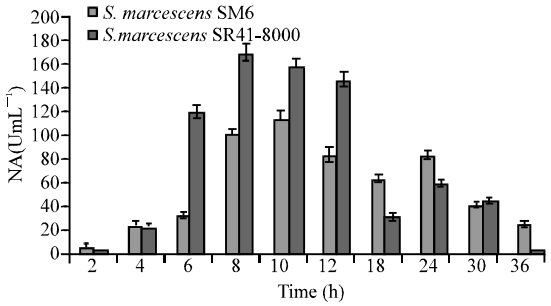

Fig. 4: Nuclease activity of wild *S. Marcescens* strains

The *S. Marcescens* nuclease is a non-specific endonuclease splitting the single and two-stranded RNA and DNA molecules (Franke, 1998).

In both wild strains nuclease activity was observed in the cell culture fluid with the maximal enzyme accumulation by the 8-10th and 24-26th h of cultivation (Fig. 4). At that in the *S. Marcescens* SR41-8000 strain the level of nuclease activity in the medium exceeded the level of activity of the *S. Marcescens* SM6 strain by 1.5 times. Thus, in the *S. Marcescens* SR41-8000 strain nuclease activity in the medium exceeded the nuclease activity of the *S. Marcescens* SM6 strain in contrast to the proteolytic activity that, on the contrary was twice as high in the *S. Marcescens* SM6 strain which reflects the peculiar features of adaptation of each strain to the specific conditions of the environment from which they were isolated.

According to the literature of sources, the *S. Marcescens* strains that are able to cause infections may be either chromogenic or pigment-free with prevailing pigment-free strains (Rosenberg, 1986). Apparently, the *S. Marcescens* SR41-8000 strain is characterized by more pronounced virulence due to a higher level of nuclease activity.

Determination of antibiotic resistance using the disk diffusion method: The Serratia kinds are resistant to a few β-lactam antibiotics: penicillin G, ampicillin, amoxicillin, amoxicillin-clavunalate, cefuroxime. All Serratia kinds are sensitive to carbapenems, though in some *S. Marcescens* strains the chromosomal gene of carbapenemase was detected (Stock, 2003). Also, *S. Marcescens* strains are resistant to nitrofurantoin (Mahlen, 2011).

The data presented in Table 1, demonstrate differences in the sensitivity of the *S. Marcescens* strains under investigation to different groups of antibacterial drugs. The maximal differences between the two strains was observed with respect to antibiotics gentamicin, amikacin, cefazoline and ampicillin. At that, *S. Marcescens* SR41-8000 features higher sensitivity to the specified antibiotics than *S. Marcescens* SM 6.

Cephalosporins and semisynthetic penicillins are referred to the extensive class of β-lactam antibiotics (β-lactams) that also include carbapenems and monobactams (Strachunsky, 2007). Resistance to penicillin antibiotics is ensured by enzymes of β-lactamases genes of which are present in the bacteria genome or are coded with plasmid genes (Oethinger, 2000).

On the basis of the data obtained by us varying sensitivity of *S. Marcescens* strains to different classes of

ABD may be related to absence in the *S. Marcescens* SR41-8000 genome of genes participating in antibiotic resistance or to the low expression thereof.

Summary: As evidences by the data obtained by us during design of the phylogenetic trees the *S. Marcescens* SR41-8000 strain is evolutionally remote from another *S. Marcescens* SM6 strain investigated by us. According to the screening performed, the *S. Marcescens* WO1-A, strain causing gourds diseases appeared to be the closet to the *S. Marcescens* SR41-8000 strain (Rascoe, 2003; Bruton, 2003).

According to the literature sources it is known that the *S. Marcescens* strains isolated from the gourd rhizosphere form a separate cluster and significantly differ from the strains of the same kind but isolated from other ecological niches by a number of metabolic and biochemical features. These differences may be represented in the gene loss or repression thereof that occurred as the result of bacteria adaptation as intracellular parasites and plant pathogens (Rascoe, 2003).

On the basis of the above-said the conclusion may be drawn that such characteristics as extracellular proteolytic and nuclease activity as well as strain sensitivity to antibacterial drugs vary for different representatives of the Serratia genus.

CONCLUSION

By means of the comparative analysis of the *S. Marcescens* strains we established that *S. Marcescens* SM6 and *S. Marcescens* SR41-8000 are not closely-related strains. By comparative analysis of proteolytic, nuclease activity and resistance of the strains being investigate to antibacterial drugs different figures were obtained for the strains SM6 and SR41-8000. In the *S. marcescens* SR41-8000 strain nuclease activity in the medium exceeded the nuclease activity of the *S. Marcescens* SM6 strain in contrast to the proteolytic activity that, on the contrary, was twice as high in the *S. Marcescens* SM6 strain. The *S. Marcescens* SR41-8000 strain features high sensitivity to different classes of antibiotics than the *S. Marcescens* SM6 strain.

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