Theses of Doctoral (PhD) Dissertation

External factor generated changes in the structure and activity of *Pseudomonas* strains

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1. Introduction

1.1 General

Pseudomonas genus contains more than 200 species, the majority of which are saprophytes living in the soil and water. They are Gram-negative; they produce no spores, being rod-shaped with a polar flagellum. They have no real capsule. They reproduce easily on agar plate. They are non-fermenter, traditional aerobic bacteria.

Among the numerous *Pseudomonas* species *P. aerugniosa* is the most studied and the most significant one in medical point of view. It is a bacterium that is able to survive and replicate at a wide range of temperatures. In great quantities it is capable of producing exopolysaccharide, which helps adhesion and hampers protection against bacteria in the host to a great extent. It is a common nosocomial pathogen; in other terms, it is a bacterium that causes infections in hospitals. Colonies smell sweet like linden-trees; their colour is either greenish due to water-soluble pyoverdin or bluish due to pyocyanin or perhaps reddish due to the production of pyorubin pigments. On blood agar plate they characteristically cause serious beta-hemolysis. *P. aerugniosa* causes disorders mainly in immunologically damaged patients. Certain virulence factors enhance the invasive ability while others have a real toxic effect, and there are some that reduce the effectiveness of the protective mechanism of the infected person. *P. aeruginosa* has several pili, which intensify the adhesive ability of the bacterium.

Among the virulence factors of *P. aeruginosa*, toxin A is significant having the same mechanism of action as diphtheria toxin besides which exoenzyme S, various proteases, elastases, hemolysins and phospholipase C play a role in pathomechanism. Due to its Gram-negative nature it possesses endotoxin which is released in large quantities, and develops its biological effect when SIRS occurs.

Medical intervention against *P. aeruginosa* is hindered by the high degree of resistance to antibiotics. It is mainly due to the low permeability of the outer membrane, to the production of β-lactamase and to the efflux pump mechanism, through which the bacterium is able to pump the drugs already passed its membrane to the external world.

1.2 The Gram-negative cell wall

The bacterial cell wall serves the purpose of the mechanical protection of the cell, of maintaining its shape against the internal osmotic pressure, of keeping away harmful substances, of supporting intake of food and secretion, and in general, of maintaining contact with the environment. One of the main components of the cell wall is peptidoglycan, which is a chain made up of N-acetylglucosamine and N-acetylmuramic acid and the bond evolving between tetrapeptides attached to the muramic acid forms a 3-dimensional mesh-like layer. The density of cross-linkages determines the strength of the structure, which is substantially thicker in Gram-positive bacteria than in Gram-negative bacteria, and generally it counter-balances the bigger internal osmotic pressure. In case of Gram-negative cells the periplasmatic space is found moving outwards of the cytoplasmic membrane that contains far fewer layers of peptidoglycan than in Gram-positive bacteria. The periplasm is essential for Gram-negative bacteria since dissolved proteins, enzymes degrading antibiotics, transferring molecules and many other proteins and enzymes enhancing the resistance capability of the bacterium can be found here.

The outer membrane forms the outermost layer of the cell wall of Gram-negative bacteria that is constructed of several components. It is a lipid bilayer; endotoxic lipopolysaccharides (LPS) and in case of certain bacteria, lipooligosaccharides (LOS) are embedded into the outer surface. The inmost component penetrating into the outer membrane of these molecules is Lipid A, which is a toxic component of the endotoxin molecule. It is a specific phospholipid that is highly similar in case of nearly all Gram-negative bacteria. Moving outwards from inwards the next component of a lipopolysaccharide molecule is the core that comprises of a separable outer and inner core. Both are highly conserved consisting of sugar molecules. The next endotoxin component attached to the core is the O-side chain. This provides the major outermost antigen of Gram-negative bacteria, the O-antigen. It is constructed of sugar molecules whose types, quantity and attaching sequence are greatly varied; therefore, it forms a number of different serogroups. The diversity of the LPS of pathogens significantly makes the self-defence of the host more difficult in case of infections.

There are numerous porin proteins in the outer membrane which form a “channel” through the membrane enabling the transportation of substances to and from the bacterial cell.

In addition to the channel proteins, the outer membrane contains a number of other proteins which are collectively referred to as outer membrane proteins (OMP).

2. Aims of Thesis

1. Our aim is to explore the changes occurring in the proteins and endotoxin of the bacterium resulting from the changes of temperature
2. Our aim is to study the effect of temperature on the invasive capability of *P. aeruginosa* into eukaryotic *P. aeruginosa.*
3. Our aim is to study the effect of clove and cinnamon essential oils on the outer membrane proteins of *P. aeruginosa.* The aim is to explore the structural changes of the bacterium as well as to identify the effective components of the essential oil.

3. Materials and methods

3.1. Bacteria

The bacterium strains used for the experiments were *Pseudomonas aeruginosa* PAO1 and *Pseudomonas aeruginosa* ATCC 27853 strains. The referred strains come from the collection of the Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Pécs.

3.2. Extraction of the outer membrane protein

The *P. aeruginosa* PAO1 strain was inoculated into 500 ml Mueller-Hinton broth and then was put into a shaking incubator for a night. Incubation took place at three different temperatures: 25, 37 and 42°C degrees, respectively. Next day the bacterium culture was centrifuged (4000 g, 60 minutes, 4°C degrees), and then the sediment was suspended in physiological saline solution. The suspension was centrifuged again with the above mentioned parameters, and this step was repeated once more. Subsequently the samples were taken in 15 ml sonication buffer, and then they were exposed to sonication, and centrifuged (6000 g, 15 minutes, 4°C degrees); following that, the supernatant was discarded for further ultracentrifugation (100.000 g, 60 minutes, 4°C degrees). Thereafter, the sediment was suspended in 5 ml 0,5 % *N*-laurylsarcosin solution, incubated at room temperature for 20

minutes, and then ultra-centrifuged again. The outer membrane proteins were located in the sediment.

3.3. Endotoxin purification

Bacteria were inoculated into 8 litre Mueller-Hinton broth, and then were put into a shaking incubator for a night (200 rpm). The culture was centrifuged at 3000 g for 40 minutes at 4°C. The supernatant was discarded and then the sediment was washed in physiological saline twice. 150 ml distilled water was added to the bacteria, and then it was mixed. The sample was placed in an ultrasonic cleaner for 2 minutes. Following that, 150 ml 90% phenol was added to the suspension, and the sample was placed in a 56ºC degrees water bath. With the discontinuance of the phenol-water phase stirring of the sample became feasible. The sample was stirred at the above designated temperature for 10 minutes, and having cooled it to 4°C degrees it was centrifuged (3000 g, 40 minutes, 4°C degrees). The water phase was separated from the phenol phase, and then again 150 ml distilled water was added, and stirred at 56°C degrees. The sample was again centrifuged with the above parameters, and the water phase was collected to the above then was dialysed in a dialysis sack for 6 days. The sample was condensed to a small volume, and was ultra-centrifuged (4 hours, 100.000 g, 4°C degrees), the sediment was suspended in distilled water, and then centrifugation was repeated for three more times. The final sediment contained the endotoxin which was freeze-dried.

3.4. Lipid A preparation

5 ml 1% acetic acid was added to 5 mg of the lyophilised endotoxin sample, and incubated at 100°C degrees in a water bath for 1 hour, and then the sample was centrifuged (8000 g, 20 minutes, 4°C degrees). Following the centrifugation the sediment in the tube contained lipid A. In order to discard the acetic acid the sample was lyophilised.

3.5. Polyacrylamide gel electrophoresis

4% stacking and 14% separating polyacrylamide gel was used, and it was run on 120-V voltage for 60 minutes. Having separated the protein samples the gel sheets were incubated in Coomassie Brilliant Blue dye solution for 14 hours. Following the incubation, the dye not bound to the protein was removed with a differentiating solution, and then it was analysed with the help of GE ImageScanner and MagicScan.

3.6. Determination of protein concentration

In order to determine the protein concentration of the samples Bio-Rad DC Protein Assay Kit was used according to the instructions of the distributor. Using a Fluostar Optima (BMG Labtech GMBH, Ortenberg, Germany) tool the concentration measuring was conducted on 675 nm.

3.7. Two-dimensional gel electrophoresis

The outer membrane protein samples extracted from the bacteria were dissolved in buffer A. To carry out the experiment 7-cm linear, 3-10 pH gradient gel strips were used. 80 µg protein was applied onto the gel strips, and then rehydrated. After that, 800 µl mineral oil was added with pipettes. The samples made up this way were incubated at room temperature for 16 hours, and then isoelectric focusing followed.

Running of the 1st dimension (isoelectric focusing) was done in 3 stages:

1, 250 V, linear, for 2 hours
2, 500 V, linear, for 2 hours
3, 4000 V, rapid, up to 10000 VH

The gels were drained off and kept in a rehydrating compartment at minus 80 C^{o} degrees for a night, and the next day they were equilibrated. Following the equilibration the strips were placed on the already prepared 12% polyacrylamide gel, and after the standard application of the protein the second dimension was executed with 120 V voltage, whose course is in accordance with the details described in the PAGE method.

3.8. Extraction of protein from gel

The extraction of protein from gel was carried out with the Shevchenko method.

3.9. MALDI-TOF mass spectrometry

Mass spectrometry measures were performed with an Autoflex II MALDI-TOF/MS tool (Bruker Daltonics, Bremen, Germany).

To identify the protein the MASCOT algorithm was applied (http://www.matrixscience.com), and the database search in the Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) was performed with the following parameters:

- carbamide-methyl (methylurea) cysteine as constant variation,
- methionine oxidation as varying modification,
- mass accuracy in case of MS measure within 3 Da,
- in case of MS-MS within 0.8 Da,
- number of the missing splicing site of the enzyme: 2

3.10. Lab-on-a-chip technique

To carry out the examinations the Agilent 2100 Bioanalyzer was used (Agilent Technologies, Palo Alto, CA, USA). Measurements and analyses were performed with the High Sensitive Protein 250 programme and its kit.

3.11. Cell invasion assays

Cell invasion assays were carried out on the cell line A549. The assay was done as described in Steele-Mortimer. Infectious plate count was 10^9**.**

3.12. Microscopy analysis of cells

Cell staining was completed based on Mileykovskaya During the microscopy analysis Olympus IX 81 microscope and Hamamatsu Orca camera was used.

3.13. Gas chromatographic analysis

The composition of essential oils was determined using a gas chromatograph (Agilent 6890N GC, 5973N) attached to the mass spectrometer. Separation was performed on capillary column HP-5MS. Identification of the components was completed based on NIST spectral library and on scientific literature data. In order to identify the percentage of the components Fisons GC 8000A gas chromatograph equipped with flame ionization detector (FID) was employed. The identification of the components of the essential oils was carried out on the basis of retention time and by adding interim standard whereas the determination of the percentage of the components was done with area normalization method.

3.14. Tube dilution method to determine the minimal bactericidal concentration of essential oils

Preparation of 5% essential emulsion: 100 µl essential oil + 40 µl 10 % polysorbate 80 (aqueous solution) + 1860 µl Mueller-Hinton liquid broth. This was homogenised with vortex. Then the preparation of the dilution line was completed in half-log dilution between 5 and 0,02 % v/v concentrations. Control tubes: a – containing only broth, b – bacterium suspension, which did not include essential oil emulsion, c – broth containing 0,2 % polysorbate 80 and bacterium suspension. 10 µl OD 600nm 1,00 bacterium suspension was added to each tube. The next step was incubation at 37 °C degrees for 24 hours, and then inoculation followed 10 – 10 µl per tube on Mueller-Hinton broth for evaluation. Then incubation followed at 37 °C degrees for 24 hours, and the final step was to read the findings by counting the colony-forming units.

4. Results

4.1. The effect of temperature on bacterial flagellin and on outer membrane proteins

4.1.1. Results of the two-dimensional electrophoresis

The *P. aeruginosa* (PAO1) cultures were incubated at 3 different temperatures (25℃, 37℃, 42℃). Proteins were isolated from the cultures, and the samples were separated by using 2-dimensional gel electrophoresis. Following the digestion from gels the following proteins were identified: in case of the 25℃-degree sample with the help of MALDI-TOF mass spectrometer: flagellin, OprF, porin. In case of the 37 ℃-degree sample: Opr86, OstA, OprD, two different kinds of flagellin, OprF, porin, hypothetical protein PA2318, lipid A 3-O-deacylase, whereas in case of the 42 ℃-degree sample: Opr86, OstA, OprD, three kinds of flagellin, OprF, porin, hypothetical protein PA2318, lipid A 3-O-deacylase. Out of the three samples the proteins of the bacterium cultured at 25℃ degrees showed the least intensity and the poorest composition. In the two higher temperature points the proteins isolated from the cultured bacteria were highly similar. In case of all three samples the porin proteins were present with various kinds among the outer membrane proteins as it could be expected. The Opr86 could not be detected from the 25 °C-degree culture of the strain. Therefore, it was concluded that lower temperatures have an effect on the outer membrane build-up and by

doing so it influence the permeability of the membrane. The other channel proteins such as the OprD or the OprF play a significant role in resistance against antibiotics. The OprD protein was only detected in the bacterium samples cultured at 37 and 42°C degrees while the OprF was found in the bacteria cultured at all three different temperatures; however, it was the most intensive following the incubation at 25°C degrees. The absence of the OstA protein plays a role in the resistance against organic solvents primarily when using disinfectants at hospitals. This protein in the gel pictures could only be found among the outer membrane proteins expressed during the incubation at 37 and 42°C degrees; therefore, it can be assumed that *P. aeruginosa* (PAO1) incubated at 25°C degrees is more sensitive to organic solvents compared to the other two incubation circumstances. The flagellin protein could be found among the detected proteins in various compositions and intensities; this protein plays a role in the build-up of bacterial flagellum.

4.2. Analysis of endotoxin samples

4.2.1. Results of the microchip method

The LPS sample isolated after the 37°C incubation resulted in significantly more intensive peaks than the LPS samples produced at the other two temperatures. The three samples showed almost the same peak numbers up to the 35th second of the experiment/measuring although the difference in intensity among the peaks is remarkable. However, 35 seconds later the signs of the 37°C sample levelled out while those of the 25°C, and more importantly, those of the 42°C started to intensify. Better separation of the peaks was visible in case of the samples incubated at higher temperatures. These results indicate a significant number of sugar O-side chains in the higher mass range. The difference in the composition of the sugar O-side chain can influence the response of the organism to infections.

4.2.2. Results of using the ESI-IT-MS method (lipid A)

The lipid A pattern of the *P. aeruginosa* (PAO1) endotoxin was exposed with the help of mass spectrometer used in ESI-IT-MS negative ion mode. The penta- and hexa-acylated components not only showed quantitative, but also qualitative differences in the lipid A of the

bacteria cultured at different temperatures. The proportion of hexa-acylated lipid A components is significantly higher under higher temperature circumstances than in case of the two other temperatures. Comparing the intensity of penta-acylated components in relation to one another within one sample it is visible that these conditions have changed in the three samples. Relying upon these findings it can be ascertained that the culturing temperature have influenced the lipid A pattern of *P. aeruginosa* (PAO1). The lipopolysaccharide component of Gram-negative bacteria plays an essential role in the relationship between the bacterium and the host cell. The lipid A component defines its hydrophobic nature, and the sugar chains responsible for the hydrophilic character can influence this relationship. The changes occurring in these parts have a role in the hydrophobicity of the bacterium; consequently, in its adhesive capability.

4.3. Analysis of the effect of the incubation temperature on the cell invasion capability of *P. aeruginosa* (PAO1)

P. aeruginosa (PAO1) is a bacterium that is able to penetrate into human eukaryote cells. The invasion capability of the bacteria was studied after having cultured them at three different temperatures - 25°C, 37°C and 42°C in the cell culture system A549. The results suggest that the invasive capability of *Pseudomonas aeruginosa* (PAO1) changes under the effect of the cultivation temperature. Compared to the samples cultured at 37 °C the samples at 25 °C were only able to penetrate into the cells in less than fifty percent, whereas the invasion results of the bacteria cultured at 42 °C reached 4 % of those incubated at the ideal 37 °C temperature. There are several reasons for this change. One of them could be the expression of the pili and proteins on the cell surface necessary for bacteria to adhere on the cells. The other reason could be the mucus excreted extracellularly, and the formation of biofilm in case of *Pseudomonas* strains. The bacterium colonies incubated at three different temperatures that were used for the analysis showed differences visible to the naked eye in their colours and morphology.

4.3.1. Microscopic examination of bacterial cell invasion

In order to prove the cell invasion caused by *P. aeruginosa* (PAO1) the cells exposed to invasion were examined with a microscope. To mark the bacteria specifically Nonyl Acridine Orange fluorescent dye was used, which specifically gets attached to the cardiolipin found in

the bacterium membrane. The findings of the microscopic examination show the bacteria at areas equal that of the eukaryote cells.

4.4 The results of the experiments carried out with essential oils

Experiments were carried out on *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC 27853 strains. The effect on the vitality of the bacterium and the changes occurring in the composition of its proteins were analysed. The chosen samples were clove and cinnamon essential oils.

4.4.1. In case of the PAO1 strain there were several differences visible in the outer membrane proteins of the bacterium following the control and the treatment with cinnamon essential oil. The treatment with 0.5-MBC essential oil changed the composition of the protein to less extent than the one with the 2-MBC dose; however, the quantitative differences could be well seen with the former sample although the protein pattern and the number of the signs are almost the same as that of the control sample. Based on the chip measurement the treatment with 0.5-MBC contraction cinnamon essential oil showed an enhanced intensity of proteins between 40 and 60 kDa compared to the control sample; furthermore, it also indicated the absence of the 80.8-kDa protein present in the control sample. The treatment with the higher concentration cinnamon essential oil changed the composition of the outer membrane proteins to a greater extent based on the chip measurement. Having compared the two measurement findings, the most spectacular difference is the absence of components. The 42.7-kDa protein cannot be detected in the treated sample, and the same applies for the 80.8-kDa protein of the control sample. Under the effect of the cinnamon essential oil treatment a significant deficiency was detected in the greater mass range as compared to the control sample. Two proteins giving a stronger sign and two others giving a weaker sign disappeared completely in the range over 140 kDa due to the essential oil treatment. The findings of the microchip method were in agreement with the results of the PAGE method. A clearly visible streak was present in the control sample in the greater mass range, which could only be detected following the treatment with 0.5-MBC cinnamon essential oil although with less intensity. The control sample contained two visible streaks in the greater mass range while the treated sample contained only one. In addition, the streak visible in approximately 35-kDa range showed stronger intensity in case of each treated sample than in the control sample. Due to the 2-MBC treatments its intensity exceeded the intensity growth induced by the 0.5 MBC.

The appearance of a firm streak could be observed directly over the streak indicating the smallest mass range. Contrary to the former protein, the intensity of this protein decreased under the effect of the 2-MBC treatments compared to the treatment with 0.5 MBC, which shows almost the same appearance as that of the control sample. The streak visible in ca. 25-kDa range shows conformity with this pattern.

4.4.2. The essential oil treatment also changed the composition of the outer membrane proteins of the *P. aeruginosa* 27853 strain. 57-kDa protein appeared in the samples only after the 0.5-MBC cinnamon essential oil treatment. Nevertheless, the 71-kDa protein disappeared from the samples due to the treatments. The most significant difference occurring under the effect of the 2-MBC clove essential oil treatment was the 38.6-kDa protein with increased intensity. The streak indicating a protein with smaller mass in the 40-kDa range exceeds the intensity of the control sample in case of each treated sample; however, it is more intensive after the cinnamon essential oil treatments. A streak indicating a protein with a bigger molecular mass also appeared in the range, which was only detected due to the 0.5-MBC cinnamon essential oil treatment. In the bigger molecular mass range there were two streaks visible in case of the control sample; however, the streak indicating the protein with smaller molecular mass out of the two could not be detected in any of the treated samples. Outside the marked ranges the gel picture showed a strong protein streak in the ca. 15-kDa molecular mass range in case of all samples. This streak showed almost the same intensity in the control samples and in those treated with cinnamon essential oil whereas it showed a less intensive appearance in case of samples incubated with clove essential oil.

3 streaks were cut out of the gels and identified with the help of MALDI-TOF. The results showed the flagellin content of the d1 streaks with the sequence coverage of 21.7 % while the dihydrolipoamide dehydrogenase enzyme was identified in the 3d2 streak with the sequence coverage of 10.7 %.

5. Conclusions, new findings

The following have been proved:

1. The culture temperature influences the pattern and intensity of the biochemical processes of *P. aeruginosa.*

1.1 With the help of 2-dimensional electrophoresis following the outer membrane protein isolation, a great number of proteins were identified, which show a diversified picture under the effect of the three cultivation temperatures. These changes affected such porin and flagellin proteins that may influence the vitality and the virulence of the bacterium as well as its resistance to antibiotics.

1.2. It was also demonstrated that the change in temperature may affect the structure of the endotoxin, which may influence the reactions triggered in the host organism since the composition of the sugar O-side chains can influence the organism's response to the pathogen. The composition of the lipid A layer may modify the penetration of certain substances, even that of antibiotics into the bacterium cell. It can be concluded that the culture conditions influence the structure of *P. aeruginosa* and as a consequence, its activity and pathogenicity as well.

1.3. Having carried out the invasion experiments temperature dependent changes were observed, which could be in relation with the change in protein composition.

2. Essential oils influence the protein composition of the outer membrane of Pseudomonas aeruginosa. We have found such proteins that appeared as a result of the treatments and some that disappeared. It was proved that essential oils in the appropriate concentration not only hinder the growth of bacteria but are able to kill them as well.

6. Publications

Makszin L, Kilár A, Felső P, Péterfi Z, Kocsis B, Kilár F (2012). Quantitative microfluidic analysis of S- and R-type endotoxin components with chip capillary electrophoresis. Electrophoresis. 33: 3351-3360. IF.: 3,261

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7. Conferences

Posters

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Makszin L, Kilár A, Felső P, Kocsis B, Kilár F.: Analysis of *Pseudomonas phyla* with microchip electrophoresis, Congress of the Hungarian Society for Separation Sciences, Tapolca 2010.

Felső P, Kilár F, Kocsis B.: Examination of the outer membrane proteins of *Pseudomonas* strains treated with different methods. International Congress of the Hungarian Society for Microbiology, Budapest 2011.

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Makszin L, Kilár A, Felső P, Péterfi Z, Kocsis B, Kilár F.: Quantitative analysis of S- and R-type endotoxin components with chip capillary electrophoresis. International Symposium on MicroScale Bioseparations and Analyses. Genf 2012.

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