

A study on the mode of action of antimicrobial plant peptides and identification of their interacting partners with the help of novel *S. meliloti* based ORFeome library

Ph.D. thesis

Kata R. Mikuláss

Supervisor: Dr. Attila Kereszt

Ph. D. School in Biology

University of Szeged

Faculty of Science and Informatics

Biological Research Centre of the Hungarian Academy of Sciences,

Institute of Biochemistry

Szeged

2016.

INTRODUCTION

Rhizobia are Gram negative bacteria that induce the formation of the symbiotic organs called nodules on the roots of compatible leguminous plants such as *Medicago*, *Lotus*, *Pisum*. In these nodules the bacteria differentiate into nitrogen fixing bacteroids. In certain legumes belonging to the Inverted Repeat Lacking Clade (IRLC) the bacteria undergo a terminal differentiation process: their size and DNA content increases via genome endoreduplication and they lose their cell division capacity. This developmental program is governed by nodule-specific cysteine-rich (NCR) plant peptides.

The NCR peptides resembling the defensin-like AMPs are produced specifically in the infected cells of the symbiotic nodules. The *NCR* genes are coding for short, 25-60 amino acid long, secreted peptides, which in mature forms contain four or six cysteine in conserved positions. The primary structures of the approx. 600 peptides identified in the *Medicago truncatula* genome show weak homology to each other and significant differences were found in their isoelectric point and expression pattern. We revealed that their primary function in the symbiosis is probably to govern the terminal bacteroid differentiation of the endosymbiotic bacteria. Because to their similarities to the defensins we examined their antimicrobial features. We found that the cationic NCR peptides with higher isoelectric point have strong activity against Gram negative and Gram positive bacteria as well as yeast-like growing and filamentous fungi.

Due to their strong inhibiting and killing activity they might replace the antibiotics that became ineffective against the multidrug resistance microorganisms. For this purpose it is very important to understand their mode of action.

During my work, we examined the mode of action of the cationic antimicrobial peptides. On the other hand, we would like to determine the role of the NCR peptides in the terminal differentiation by creating a new library to investigate the putative bacterial interactors of the symbiotic plant peptides.

OBJECTIVES

For these purposes we planned the following experiments:

1. The determination of the effect of the NCR peptides on the integrity of the outer and inner membranes as well as the membrane potential of bacteria in order to reveal the mode of action of the cationic peptides with antimicrobial activity.
2. The examination of the NCR peptides treated bacteria by SEM and AFM.
3. The establishment of a new yeast two-hybrid compatible *S. meliloti* ORFeome library for the determination of the interacting partners.
4. The optimization of the use of the new ORFeome library.
5. Identification of the bacterial interacting partners of NCR247.
6. Confirmation of the results of the Y2H screen by other biochemical techniques (pull-down, coIP).

METHODS

1. Methods used during the examination of the mode of action of the cationic peptides

Measuring the integrity of the outer membrane by NPN fluorescence

The effect of the cationic peptides on the outer membrane integrity was measured with the help of the 1-N-phenyl-naphthylamine (NPN) fluorescent dye. The peptide treatment was carried out in a 96-well microtiter plate, where the change of the fluorescence intensity was measured in a FLUOstar OPTIMA plate-reader (BMG Labtech). After three (9 second) cycles, the peptides were added to the samples and the fluorescence were measured for an additional 18 cycles.

Measuring the integrity of the inner membrane by β -galactosidase activity

The rate of the inner membrane permeabilization effect of the cationic peptides was determined with the help of the β -galactosidase enzyme and its membrane impermeable artificial substrate, ortho-Nitrophenyl- β -galactoside (ONPG). The membrane disruption and, thus, the enzymatic cleavage of the substrate was followed by spectrophotometry. The measurements were performed as described by Miller. The β -galactosidase activity was calculated from the absorbance (A₄₂₀), time of the treatment (t), the volume of the samples (V), the amount of the treated cells (OD₆₀₀) (Miller unit: $(A_{420}/t * V * OD_{600}) * 1000$). The loss of membrane integrity was followed by the measurement of the fluorescence of the membrane impermeable DNA-binding dye, propidium iodide.

Measuring the membrane potential after the peptide treatment

For the measurements, the BacLight™ Bacterial Membrane Potential Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. The NCR peptide induced fluorescence changes were characterized by shifts in the red/green ratio. The effect

of the peptide on the membrane potential was compared to the effect of the proton ionophor CCCP (carbonilcyanid m-chlophenilhidrazone).

Atomic Force Microscopy (AFM)

For the measurements, freshly cleaved 1*1 cm mica (SPI-Chem™ Mica Sheets, Structure Probe, Inc., West Chester, PA, USA) surfaces were used as supports for the bacteria. AFM measurements were carried out with an Asylum MFP-3D head and controller (Asylum Research, Santa Barbara, CA, USA). The driver program MFP-3DXop was written in IGOR Pro Software (version 6.22a, Wavemetrics, Lake Oswego, OR, USA).

Height and amplitude images of the same cells were taken before and after the peptide treatment.

Scanning electron microscopy

Exponentially growing cultures of bacteria were collected, washed twice and suspended 10 mM potassium phosphate buffer (pH=7) and treated with peptides at 30 °C for 30 minutes. After peptide treatment, the cells were fixed with 2.5% glutaraldehyde dissolved in cacodylate buffer, washed with 10 mM potassium phosphate buffer (pH=7) and were dehydrated in ethanol of increasing concentration (50%-70%-80%-90%-95%-98%-100%). As the last step of the dehydration, the 100% ethanol was replaced by tert-butanol in which the cells were kept at 4 °C overnight. The samples were freeze-dried, covered by gold and investigated with a HITACHI S-4700 scanning electron microscope.

2. Methods used during the determination of the interacting partners of NCR peptides

Yeast cells and growing conditions during the yeast two-hybrid experiments

Saccharomyces cerevisiae AH109 (Clontech Matchmaker™ Gold Yeast Two-Hybrid System) was used during the yeast two-hybrid experiments. We optimized the production For the preparation and transformation of competent yeast cells published methods

optimized in our laboratory were used. The transformation efficiencies were determined with the help of leucine and tryptophan free yeast minimal medium (SD-LT, Clontech). The clones carrying genes coding for interacting partners were selected on histidine, leucine and tryptophan free (SD-HLT) as well as adenine, histidine, leucine and tryptophan free (SD-AHLT) minimal media (Clontech).

The establishment of a new yeast two-hybrid compatible *S. meliloti* ORFeome library for the identification of protein-protein interactions

We modified the classic yeast two-hybrid prey vector pGADT7-AD with the insertion of the RfB cassette the Gateway Vector Conversion System (Life Technologies). We used an optimized version of Gateway LR reaction for the recombination of all the predicted genes of the *S. meliloti* present in the ORFeome library into the modified pGADT7GW-AD vector.

Determination of the protein-protein interactions using the newly created ORFeome library

After the optimized transformation steps and plating the colonies onto different selective medium, the colonies were inoculated into 96-well microtiter plates. The growing cultures were transferred onto different selective media by a replica plate technique, which created opportunities to work with numerous yeast cells during the examination. To determine the weak and strong interaction partners, the colonies from the different selective media were examined by colony PCR followed by the sequencing of the amplified DNA fragments.

RESULTS

In the frame of the presented work, we investigated the mode of action of the antimicrobial NCR peptides and identified putative interacting protein partners of the NCR247 from *Sinorhizobium meliloti* 1021.

1. Determination of the effect of the NCR peptides on the integrity of the outer and inner membrane well as the membrane potential of bacteria.

The dose-dependent membrane disruptive effect of the cationic peptides on the outer membrane (OM) was measured with the hydrophobic fluorescent probe 1-N-phenyl-naphthylamine (NPN), which cannot enter the intact OM, but can pass the destabilized one. By entering the phospholipid layer, the dye gives rise to strong fluorescence. The control Polymyxin B, which destabilizes the outer membrane structure due to its cationic amino acids, caused quick and strong fluorescence. The cationic NCRs caused also the quick increase of fluorescence, however, its extent was not as high as in the case of Polymyxin B. Based on these results we can conclude that the cationic NCR peptides can destabilize the outer membrane to a certain extent but it is not the primary reason of the death of the treated cells. The anionic or weakly cationic peptides did not show any membrane disruptive activity.

To investigate the possible damage to the inner membrane (IM) caused by the cationic peptides we took advantage of the fact that the IM is not permeable for the artificial substrate of the cytoplasmic β -galactosidase enzyme. Thus, the enzyme activity can be measured only after the disruption of the membrane. Treatment of *S. meliloti* cells expressing constitutively the lacZ gene with the cationic peptides resulted in measurable β -galactosidase activity that was 2–20% of the total enzyme activity obtained after disrupting the cells with SDS and chloroform. The anionic peptides didn't have membrane disruptive features.

The IM damage caused by the cationic peptides was confirmed by detecting the loss of the membrane potential of the peptide treated cells with the help of the fluorescent membrane-potential indicator dye, DiOC₂(3), provided in the BacLight™ Bacterial Membrane Potential Kit. Thus we concluded that the primary reason for the

antimicrobial activity of the cationic peptides is the elimination of the membrane potential.

2. The examination of the NCR peptides treated bacteria by SEM and AFM.

The changes induced by the cationic peptides in the surface and the shape of the bacteria were determined by atomic force and scanning electron microscopic studies, which revealed differences in the mode of action of the examined cationic peptides, too. Some of these peptides caused the aggregation of the bacterial cells and the formation of thread-like structures that seem to be formed by materials released from destabilized cell surfaces, and some others cause the excretion of the content of the bacterial cells

3. The establishment of a new yeast two-hybrid compatible *S. meliloti* ORFeome library for the determination of the interacting partners.

The yeast two-hybrid system provides an opportunity to identify the intracellular interacting partners, targets of the examined cationic peptides. During my work, we transferred the ORFeome library containing all the predicted genes of *S. meliloti* 1021 into a newly created prey vector, which is compatible with the classic yeast two-hybrid system. During the construction and testing of the new system, we established a cost-efficient Gateway (LR) recombination protocol and a more effective yeast transformation method. The efficiency of the *en masse* recombination was confirmed by sequencing of selected pools.

4. Optimization of the use of the new ORFeome library.

To test the functionality of the library, pairwise investigations and mini screens were performed with proteins known, to form homodimers (cI repressor of rhizobiophage 16-3) homotetramers (Hfq) or hetero-complexes (such as FixA and FixB, the putative α and β subunits of an electron transport flavoprotein complex), such as HfQ,

FixA and FixB, and cI protein of phage 16- 3 of *S. meliloti* strain Rm41. In all cases we could detect the interaction as we suspected.

5. Determination of the bacterial interaction partners of NCR247.

After setting up the new system, we initiated the identification of the targets of the most investigated NCR247 peptide and could isolate strong and weak interacting partners. Interestingly, on the most selective medium we could detect only one interaction partner, which is the endopeptidase PepF (SMc04012). Additionally we could find more than 200 weak interaction partner of the NCR247 peptide, too.

6. Confirmation of the results of the yeast two-hybrid screen by other biochemical techniques (pull-down, coIP)

The interaction between the peptide and a few putative interactors were proved by other biochemical techniques such as immunoprecipitation and pull-down analysis. From the weak interaction partners we selected the RNA polymerase sigma factor FecI (SMc04203). It is known that the expression of the *fecI* gene is regulated by NCR247 peptide. In accordance with the results of the Y2H system, we could confirm the interaction between the NCR247 and the HA tagged FecI protein by immunoprecipitation.

The strong interaction between the His-tagged PepF endopeptidase and NCR247 was confirmed by pull-down analysis.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATION

Kata R. Mikuláss, Krisztina Nagy, BalázsBogós, ZsoltSzegetes, EtelkaKovács, Attila Farkas, GyörgyVáró, ÉvaKondorosi and Attila Kereszt (2016) „**Antimicrobial nodule-specific cysteine-rich peptides disturb the integrity of bacterial outer and inner membranes and cause loss of membrane potential**” *Ann Clin Microbiol Antimicrob* 15:43

IF₂₀₁₅:2,083

ADDITIONAL PUBLICATIONS

Van de Velde, W., G. Zehirov, A. Szatmari, M. Debreczeny, H. Ishihara, Z. Kevei, A. Farkas, K. Mikulass, A. Nagy, H. Tiricz, B. Satiat-Jeunemaitre, B. Alunni, M. Bourge, K. Kucho, M. Abe, A. Kereszt, G. Maroti, T. Uchiumi, E. Kondorosi és P. Mergaert (2010). "**Plant Peptides Govern Terminal Differentiation of Bacteria in Symbiosis.**" *Science* 327(5969): 1122-1126.

IF₂₀₁₀: 31.364

Nagy K, Mikuláss KR, Végh AG, Kereszt A, Kondorosi É, Váró G, Szegetes Z (2015). „**Interaction of cysteine-rich cationic antimicrobial peptides with intact bacteria and model membranes.**” *Gen Physiol Biophys.* 34(2):135-44

IF₂₀₁₅: 0,875