**Magdalena Płotka**

*The field of* ***Natural sciences***
*The discipline of* ***Biological sciences***

# Molecular analysis of bacteriophage and bacterial lytic enzymes showing similarity to eukaryotic peptidoglycan recognition proteins

---

Summary of Professional Accomplishments

Laboratory of Extremophiles Biology, Department of Microbiology, Faculty of Biology, University of Gdansk

**Gdansk 2020**

1. **Name:** Magdalena Płotka

2. **Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation:**

2007 PhD in Biological Sciences in the field of Biochemistry obtained at the Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk. My doctoral dissertation is entitled "Protein components of the mitochondrial nucleoid of yeast *Saccharomyces cerevisiae*"; supervisor: Prof. dr hab. Jaroslaw Marszalek, Department of Molecular and Cellular Biology, Laboratory of Evolutionary Biochemistry.

1999 MSc in Biotechnology; Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk; My thesis is entitled „ Lack of correlation between the A1 / A2 polymorphism of the GPIIIa gene and the incidence of myocardial infarction in the population of Northern Poland"; supervisor: Prof. dr hab. Janusz Limon, Department of Biology and Genetics, Medical University of Gdansk.

3. **Information on employment in research institutes or faculties/departments or school of arts:**

from 2012 Department of Microbiology, Faculty of Biology, University of Gdansk; positions: 2012-2014 Senior specialist, od 2014 – up to now Adjunct

2010 – 2011 INVICTA Fertility Clinics and Medical Diagnostic Laboratories, Gdansk Science and Technology Park, Pomeranian Special Economic Zone LTD, Gdansk; position: Assistant at the Laboratory of Molecular Biology and Cytogenetics

2007-2010 Nuffield Department of Women's & Reproductive Health, Medical Science Division, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford; Prof. Joanna Poulton group, position: Postdoctoral research assistant

2003-2007 Doctoral Studies, Intercollegiate Faculty of Biotechnology UG&MUG; PhD student

1999-2003 Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Medical University of Gdansk; position: Assistant professor

## 4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act:

Scientific achievement and the points received for it according to the article 219 paragraph 1 point 2 of the Act on Academic Degrees and Titles and on Degrees and Titles in Art is a series of 5 topically related publications on functional and structural characterization of bacterial and bacteriophage derived lytic enzymes. The cycle contains 5 original works. These works were published between: 2014-2020. They cover both basic research and application studies of lytic enzymes that show primary sequence similarity to eukaryotic peptidoglycan recognition proteins. These publications are the result of interdisciplinary collaboration with academic and research institutions as well as with biotechnology companies both in Poland and abroad.

The total value of the Impact Factor (IF) of the works constituting the achievement is **19,095**. The total number of points awarded by MNiSW (Ministry of Science and Higher Education Republic of Poland) for the works constituting the achievement is **580** according to the Appendix to the Announcement of the Minister of Science and Higher Education, 18th December 2019.

### a) The title of the scientific achievement:

Molecular analysis of bacteriophage and bacterial lytic enzymes showing similarity to eukaryotic peptidoglycan recognition proteins.

### b) The publications included in the scientific achievement:

**4.1 Plotka M.**, Kaczorowska A.K., Stefanska A., Morzywołek A., Fridjonsson O.F., Dunin-Horkawicz S., Kozlowski L., Hreggvidsson G.O., Kristjansson J.K., Dabrowski S., Bujnicki J.M., Kaczorowski T.: Novel highly thermostable endolysin from *Thermus scotoductus* MAT2119 bacteriophage Ph2119 with amino acid sequence similarity to eukaryotic peptidoglycan recognition proteins. Applied and Environmental Microbiology 80(3), 2014, p. 886-895. (**$IF_{2014}$ 4,251; $MNiSW_{2014}$ = 40**).

**4.2 Plotka M.**, Kaczorowska A.K., Morzywolek A., Makowska J., Kozlowski L.P., Thorisdottir A., Skirnisdottir S., Hjorleifsdottir S., Fridjonsson O.H., Hreggvidsson G.O., Kristjansson J.K., Dabrowski S., Bujnicki J.M., Kaczorowski T.: Biochemical characterization and validation of a catalytic site of a highly thermostable Ts2631 endolysin from the *Thermus scotoductus* phage vB_Tsc2631. PLoS One 10:e0137374, 2015. (**$IF_{2015}$ 3,057; $MNiSW_{2015}$ = 40**).

**4.3 Plotka M.***, Sancho-Vaello E., Dorawa S., Kaczorowsk, A.K., Kozlowski L.P., Kaczorowski T.*, Zeth K.: Structure and function of the Ts2631 endolysin of *Thermus scotoductus* phage vB_Tsc2631 with unique N-terminal extension used for peptidoglycan binding. Scientific Reports 9:1261; 2019; https://doi.org/10.1038/s41598-018-37417-6 1. **($IF_{2019}$ 4,120; $MNiSW_{2019}$ = 40).**
* - correspondence author

**4.4 Plotka M.***, Kapusta M., Dorawa S., Kaczorowska A.K., Kaczorowski T.*: Ts2631 endolysin from the extremophilic *Thermus scotoductus* bacteriophage vB_Tsc2631 as an antimicrobial agent against Gram-negative multidrug-resistant bacteria. Viruses

11(7), 657; 2019; https://doi.org/10.3390/v11070657. **(IF$_{2019}$ 3,816; MNiSW$_{2019}$ = 30;** according to the new MNiSW score **100). *** - correspondence author

**4.5 Plotka M.*,** Szadkowska M., Håkansson M., Kovačič R., Al-Karadaghi S., Walse B., Werbowy O., Kaczorowska A.K. and Kaczorowski T.*: Molecular characterization of a novel lytic enzyme LysC from *Clostridium intestinale* URNW and its antibacterial activity mediated by positively charged N-terminal extension. Int. J. Mol. Sci. 2020, 21(14), 4894; https://doi.org/10.3390/ijms21144894. **(IF$_{2020}$ 4,556; MNiSW$_{2020}$ = 140).** * - correspondence author

Co-author statements of the publications determining the individual contribution of each author in the creation of each publication is provided in the **Appendix 5**. The statements of the person submitting the postdoctoral thesis relating to the work performed can be found in **Appendix 4, point I.2**.

**c) The discussion of the scientific objective of the above-mentioned work and the results achieved, together with a discussion of their application:**

The World Health Organization (WHO) warns that the antibiotic-resistant bacteria may be responsible for more than 10 million deaths by 2050 and if no action will be undertaken, the number will outcompete cancer-related deaths (J. O' Neill, 2016). For the first time the term antibiotic was introduced by the microbiologist Salman Waksman who described the organic substance produced by microorganisms with ability to kill or to inhibit growth of other microorganisms. The strategy applied by Waksman group allowed mining natural antibiotics from soil-derived actinomycetes. However, the application of Waksman methodology increasingly led to the rediscovery of the same classes of antibiotics, and for more than 50 years, in medicine, their new classes appear extremely rarely (Lewis, 2012). In the era of still growing number of antibiotic-resistant bacteria the scientist constantly search for new antibacterial agents as an alternative to the conventional antibiotics. Apart, from the phage therapy, nowadays experiencing its renaissance, the peptidoglycan hydrolases seems to be promising agents used to combat bacterial infections.

Peptidoglycan (PG) is a major structural component of the cell wall of both Gram-negative and Gram-positive bacteria (Vollmer et al., 2008a). The main function of PG is to maintain the cell integrity by withstanding the high intracellular turgor pressure. The polymer consists of long glycan strands: alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by β-1,4-glycosidic bonds and short stem peptides attached by a peptide bond to the MurNAc. The basic stem peptide sequence is L-Ala-γ-D-Glu-mezo-2,6-diaminopimelic acid (or L-Lys)-D-Ala. The so-called DAP type peptidoglycan is present in most Gram-negative bacteria, *Mycobacterium* and bacteria of the genera *Bacillus* and *Clostridium*, whereas the Lys type in found in most Gram-positive bacteria. In bacteria from the *Thermus* group, there is the A3β type of peptidoglycan, characterized by the presence of L-ornithine (L-Orn) in the third position of the peptide chain. In the peptidoglycan maturation process the last D-Ala is removed by D-alanyl-D-alanine carboxypeptidase. Adjacent stem peptides may be joined by a transpeptide bond between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid at position 3 or

a transpeptide bridge (for example pentaglycine bridge in case of *Staphylococcus aureus*) (Vollmer et al., 2008a).

Peptidoglycan hydrolases are enzymes which according to the substrate specificity can be classified as: 1,4-β-N-acetylmuramidases (lysozymes), endo-β-N-acetylglucosaminidases, that catalyze the hydrolysis of glycosidic bonds or endopeptidases and N-acetylmuramoyl-L-alanine amidases that target the peptide moiety (Borysowski et al., 2006). A separate group is formed by lytic transglycosylases (e.g. the product of the R gene of λ phage) that cleave the glycan chain of peptidoglycan without the involvement of water molecule.

Based on their origin (bacterial or bacteriophage proteins) and role PG hydrolases can be classified as endolysins, exolysins and/or autolysins (Schmelcher et al., 2012).

Endolysins are enzymes produced by bacteriophages (also known as phages) at the end of their lytic cycle to degrade the cell wall of the infected bacteria to release the progeny phage to the environment (Cahill & Young 2019). The first steps in phage lysis involve a temporally controlled permeabilization of the cytoplasmic membrane followed by enzymatic degradation of the peptidoglycan. One of the system of dsDNA phages of Gram-negative hosts is the holin-endolysin pathway. Holins are small membrane proteins that forms micron-scale holes in the inner membrane, releasing active endolysin into the periplasm to degrade the peptidoglycan. The term "endolysin" means lysis of bacteria from within. In 2001 in the laboratory of Prof. Vincent Fischetti for the first time purified PlyC endolysin of streptococcal bacteriophage C1 was used exogenously to eradicate group A streptococci of colonized mice (Nelson et al., 2001). Since then, as a result of joint effort of scientific community seven preclinical or clinical lysin projects are presently conducted and the first lysins targeting *S. aureus* (CF-301, SAL200, P128, and Staphefekt™) are currently being evaluated in clinical trials (Gerstmans et al., 2020).

Most importantly, because endolysins target chemical bonds of peptidoglycan, and not the metabolically active cells the presence of bacteria resistant to them is extremely rare (Love et al., 2018).

The scientists face the problem with Gram-negative bacteria, as in their case the outer membrane is an impermeable barrier that prevents endolysins from reaching the peptidoglycan layer, and only small molecules (<600 Da) pass through the porins located in the outer membrane (Gutierrez and Briers, 2021). Because four of the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.)* are Gram-negative bacteria, scientists decided to solve the problem of outer membrane impermeability using two approaches: (*i*) by fusion of endolysins with peptides capable of penetrating the outer membrane, (*ii*) by chemical or physical agents weakening the outer membrane along with endolysins. Surprisingly, some endolysins have a natural ability to pass through the outer membrane. This rare phenomenon is most often associated with the presence of an amphipathic α-helix in the C-terminal part of the protein (Thandar et al., 2016; Peng et al., 2017). An example of the protein with such intrinsic activity is the lysozyme of the T4 bacteriophage (Gutierrez and Briers, 2021).

The next group of antibacterials are exolysins (known also as bacteriocyns). Exolysins are secreted by bacteria to kill bacteria of different species or even strains within the same species. The most known example is lysostaphin, a bacteriocin secreted by *Staphylococcus simulans* biovar *staphyloliticus*. Lysostaphin is an endopeptidase that hydrolyzes the peptide bond between the third and fourth glycine residues of the pentaglycine bridge in the PG of *S. aureus* including methicyllin-resistant (MRSA) strains (Bastos et al., 2010).

Representatives of the third group, autolysins, cleave the peptidoglycan of bacteria that produce them. They are mainly involved in cellular processes, including cell growth and division, cell-wall turnover and peptidoglycan maturation. The activity of these enzymes must be tightly regulated to avoid lysis of the host cells (Vollmer et al., 2008b).

Endolysins, exolysins, as well as autolysins may share the same specificity towards peptidoglycan and may have similar spatial architecture (Vermassen et al., 2019).

The results of research on lytic enzymes presented within this work are part of global efforts to find alternative therapies to infections caused by dangerous pathogens from the group of Gram-negative bacteria.

A. **Novel highly thermostable endolysin from *Thermus scotoductus* MAT2119 bacteriophage Ph2119 with amino acid sequence similarity to eukaryotic peptidoglycan recognition proteins.** Applied and Environmental Microbiology 80(3), 2014, p. 886-895.

During the project "Exgenome molecular enzymes" granted within the European Union's Seventh Framework Programme in 2012, we received the results of bioinformatics analysis of genomes of bacteriophages isolated from the hot springs of Iceland (in collaboration with MATIS, Reykjavik, Iceland). The *in silico* analysis was performed by Dr Łukasz Kozłowski and Dr Stanisław Dunin-Horkawicz from the laboratory of Prof. Janusz Bujnicki. On the list of 32 open reading frames (ORFs) encoding putative proteins involved in the DNA metabolism the most interesting to me was the ORF encoding potential lysozyme (designated as Ph2119). Preliminary analysis of the amino acid sequence of this protein composed of 155 amino acids suggested similarity to eukaryotic peptidoglycan recognition proteins. I started to optimize the *ph2119* gene expression to purify and characterize the Ph2119 enzyme **(publication 4.1)**. I planned all practical experiments included in the manuscript. The Ph2119 lysozyme is derived of MAT2119 bacteriophage infecting thermophilic bacterium *Thermus scotoductus*. The sample was taken at Hrafntinnusker, which is located in the highlands of Iceland north of the glacier Mýrdalsjökull, where the Katla volcano is positioned. On the basis of available nucleotide sequence of MAT2119 bacteriophage genome I synthetized the *ph2119* gene in the GeneArt Gene Synthesis Service (Life Technologies) and cloned the synthetic gene into the pET15b vector (Novagen). **I have proposed the strategy, and performed the optimization of the overproduction, purification and biochemical characterization of the recombinant enzyme**. Despite the fact that lysozymes might be toxic to producing them cells, Ph2119 is the first endolysin of *Thermus* bacteriophage that was purified in mg quantity (purification yield was 24 mg per 1 liter of bacterial cell culture). The purification efficiency of the only one partially characterized φIN93 endolysin of *Thermus aquaticus* TZ2 bacteriophage was 0.0072 mg per 600 ml of bacterial cells lysate (Matsushita & Yanase, 2008).

BLASTP (Protein Basic Local Alignment Search Tool) analysis revealed similarity of Ph2119 endolysin to T7 and T3 phage lysozymes, but only at the level of 22% and 23%, respectively. What interesting, neither a partially characterized lytic enzyme from *T. aquaticus* TZ2 phage φIN93 or lytic enzymes of other *Thermus* phages show any similarity in amino acid sequence to Ph2119. Instead, bioinformatics analysis revealed similarity of Ph2119 to

eukaryotic peptidoglycan recognition proteins PGRPs that take part in innate immunity and are present from insects to mammals (Fig. 1).

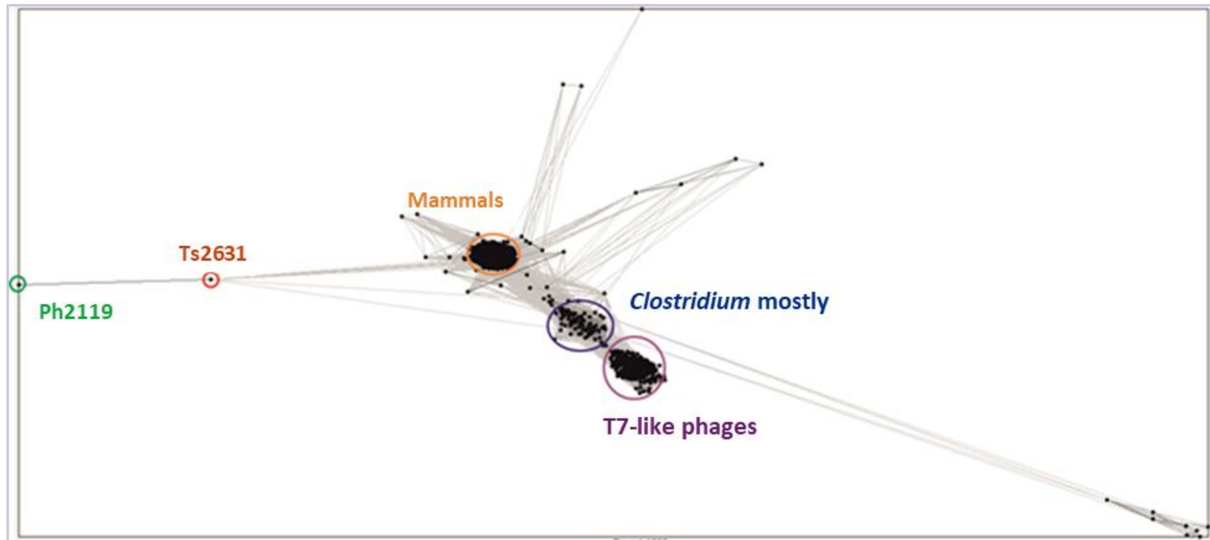


Fig. 1. Similarity of the primary sequence of the Ph2119 endolysin (marked in green) to the available protein sequences of mammals, bacteria and viruses visualized by CLuster ANalysis of Sequences (CLANS) (Frickey and Lupas 2004), modified after Prof. Kornelius Zeth. The Ph2119 endolysin shares 74% sequence identity with the Ts2631 endolysin (publication 4.2) (red) and shares similarity with mammalian peptidoglycan recognition proteins (PGRPs). Interestingly, endolysin is similar to the *E. coli* T7 lysozyme, but doesn't resemble any potential lytic protein from thermophilic bacteriophages.

After the literature search and the analysis of the amino acid sequence of the enzyme I realized the wrong annotation of the protein as a lysozyme (also known as N-acetylmuramidase or simply muramidase). The lysozyme (muramidase) hydrolyses the β(1-4) glycosidic bond between residues of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in glycan part of the peptidoglycan. BLASTP analysis revealed that Ph2119 is N-acetylmuramoyl-L-alanine amidase. The protein has a catalytic centre characteristic for type 2 amidases (the zinc ion is coordinated by two histidines and one cysteine). Despite the preliminary denomination of Ph2119 enzyme as a lysozyme, following the *in silico* analysis, I have started to use the proper term amidase or endolysin (as it is a bacteriophage protein) in further research. Similarly, T7 lysozyme that is N-acetylmuramoyl-L-alanine amidase is wrongly called lysozyme from historical perspective.

Ph2119 endolysin was the first lytic enzyme characterized in the Department of Microbiology. On the basis of available literature I optimized and applied the standard operating procedures to analyse the lytic activity of the enzyme. **Ph2119 endolysin is the first N-acetylmuramylo-L-alanine amidase from *Thermus* bacteriophage with experimentally validated lytic activity.** I revealed the optimal working conditions as pH, NaCl concentration or the optimal working temperature of the enzyme**. For the first time I have shown the lytic activity of endolysin of thermophilic bacteriophage against peptidoglycan of mesophilic Gram-negative bacteria.** In case of Ph2119 endolysin among the susceptible bacteria were: *Escherichia coli*, *Serratia marcescens*, *Pseudomonas fluorescens* and *Salmonella enterica* serovar Panama. The outer membrane of Gram-negative bacteria was permeabilized to facilitate the endolysin to get access to peptidoglycan (**publication 4.1**). Moreover, I revealed the unique

thermostability of the enzyme. **The results shown in the publication broaden the knowledge about, so far neglected, endolysins derived from bacteriophages of extremophilic bacteria**. Despite the high number (hundreds or thousands) of characterized endolysins isolated from bacteriophages of mesophilic bacteria, in the literature there were only two thermophilic endolysins described: the putative lysozyme of the ϕIN93 bacteriophage from *Thermus aquaticus* TZ2 (Matsushita & Yanase, 2008) and the lytic enzyme GVE2 from bacteriophage of *Geobacillus sp.* (Ye & Zhang, 2008).

**B. Biochemical characterization and validation of a catalytic site of a highly thermostable Ts2631 endolysin from the *Thermus scotoductus* phage vB_Tsc2631.** PLoS One 10:e0137374, 2015.

The second lytic enzyme that attracted my attention was Ts2631 endolysin derived from vB_Tsc2631 bacteriophage of *Thermus scotoductus* MAT2631. The endolysin shows 76% of primary sequence identity to Ph2119 endolysin of bacteriophage MAT2119 also infecting *Thermus scotoductus* bacteria. We received the pJOE3075 vector with cloned *ts2631* endolysin gene from MATIS Company, Iceland. However, the preliminary tests and protein overproduction trials were not successful. Therefore, I cloned the *ts2631* gene into pET15b vector (Novagene). I am the author of the conception of the work and I planned all the experiments included in the presented publication. The high level of gene expression in overproducing cells and protein solubility in cell lysate after sonication allowed for successful Ts2631 endolysin purification with use of metal affinity chromatography IMAC. Using the turbidity reduction assays (TRA) I spectrophotometrically measured decreases in the turbidity of suspensions of chloroform-treated (outer membrane-permeabilized) model substrate *Thermus thermophilus* HB8 in order to test the lytic activity of the enzyme in the contest of pH, NaCl concentration and temperature. **I experimentally validated the dependence of the lytic activity of the enzyme on the presence of divalent metal ions especially $Zn^{2+}$.** I also analysed the thermal stability of the Ts2631 endolysin after prolonged incubation at 95 °C showing **high thermoresistance of the enzyme**. The parameter that precisely describes the thermostability of the protein is the meting temperature Tm, in which 50% of the protein is in unfolded state. Determination of the Tm allows for discrimination between real thermal stability of studied protein from its thermal resistance, where the enzyme denatures at higher temperatures but after lowering the temperature its returns to its folded state and to partial or full activity (Briers et al., 2007). The Tm parameter is often used to compare thermal stability of proteins from thermophilic and mesophilic organisms (Kumar et al., 2000). In collaboration with Dr hab. Joanna Makowska, prof. UG, with use of differential scanning calorimetry (DSC) we measured the Tm = 99.82 °C of Ts2631 endolysin. **High Tm ranks the Ts2631 endolysin among the most thermostable enzymes known today. Moreover, the novelty of conducted research is the functional analysis of the catalytic residues.** I performed the site-directed mutagenesis of *ts2631* gene in order to achieve five Ts2631 variants with substitution of single amino acids. The residues for mutagenesis have been chosen based on the results of the BLASTP analysis and data available about T7 lysozyme, the most similar to Ts2631 endolysin in terms of amino acid sequence and the spatial structure (Cheng et al., 1994). The activity tests of the enzyme substitution variants performed under my supervision with MSc. student Agnieszka Morzywolek showed that all of the substitutions greatly reduced the endolysin activity. The substitutions variants of the enzyme also showed lower stability at higher temperatures.

**The Ts2631 endolysin is the first from thermophilic bacteriophage whose functionality of the catalytic centre has been experimentally confirmed.**

C. **Structure and function of the Ts2631 endolysin of *Thermus scotoductus* phage vB_Tsc2631 with unique N-terminal extension used for peptidoglycan binding.** Scientific Reports 9:1261; 2019.

Endolysins represent a class of enzymes with huge structural diversity. Most endolysins from phages that infect Gram-negative bacteria are small globular proteins generally composed of only a single catalytic domain (also known as enzymatically-active domain, EAD). Endolysins of bacteriophages of Gram-positive background often have a characteristic modular architecture with single or multiple catalytic domains (EADs) and one or more cell wall-binding domains (CBDs). There are 24 different types of EADs and 13 of CBDs with 89 different possible architectural organizations (Oliveira et al., 2013). Endolysins of bacteriophages infecting Gram-negative bacteria generally don't have the CBD that binds the enzyme to the substrate. Therefore, interesting to me was **how the thermophilic Ts2631 endolysin, which doesn't contain the CBD, binds peptidoglycan.** I was the author of the work conception and I planned all experiments included in the publication. Together with Prof. Tadeusz Kaczorowski we established the collaboration with Prof. Kornelius Zeth (current affiliation at the Roskilde University, Roskilde, Denmark) to determine the structure of the Ts2631 endolysin and to link together functional and structural studies. I optimized the enzyme's purification procedure to achieve high concentration of the protein (above 10 mg/ml) without addition of stabilizing agents such as glycerol or NaCl. The collaborative work led to structure determination of the Ts2631 endolysin deposited in the Protein Data Bank (PDB) under the accession number 6FHG. **The Ts2631 endolysin is the first from thermophilic bacteriophage with a known spatial architecture.** Structural data showed that the enzyme may form a dimer, however the experiments based on size exclusion chromatography (SEC) and analytical ultracentrifugation, together with a detailed *in silico* analysis pointed towards the monomer as a dominant form in a solution. Bioinformatics analysis performed by me, Prof. Kornelius Zeth and the multiple alignment of the amino acid sequence of Ts2631, eukaryotic peptidoglycan recognition proteins (PGRPs) and endolysins of *E. coli* phage T7, T3 and *Klebsiella* phage K11 performed by Anna Kaczorowska, PhD (publication 4.2) led to the construction, subsequent overproduction and purification of nineteen Ts2631 endolysin variants, where conserved residues were substituted by alanine (alanine screen). The substitution variants were: H31A, T32A, A33G, P54A, Y60A, R64A, D65A, R67A, Y69A, K70A, L72A, I79A, C80A, N85A, G95A, D96A, N133A, V135A and E138A. Having in hand five previously constructed, expressed and purified variants in catalytic centre of the enzyme I had 24 substitution variants of Ts2631 endolysin to determine the contribution of a specific residue to the stability and function of the protein. First, I tested the lytic activity of purified variants in comparison to the native enzyme. **The original contribution of the work is the determination of amino acids essential for the enzymatic activity of the enzyme.** Except residues of catalytic centre, necessary to maintain the lytic function of the protein were six residues: histidine (H32), threonine (T32), tyrosine (Y60), lysine (K70), cysteine (C80) and asparagine (N85). The substitution of these residues by alanine did not change the conformation of the enzyme, what was confirmed by circular dichroism (CD) analysis. Next, I adopted and optimized the methodology used previously to isolate peptidoglycan of Gram-negative *E. coli* to isolate this structure from *T. thermophilus* HB8 cells. Moreover, for the first time, to check the endolysin interaction with the substrate I applied the

procedure used previously to test substrate specificity of eukaryotic PGRPs (Yoshida et al., 1996). The analysis was performed by me with a MSc. student Sebastian Dorawa under my supervision. Obtained results showed lack of peptidoglycan binding in case of two Ts2631 endolysin substitution variants: Y60A and K70A. In the TRA analysis both of the variants showed decreased lytic activity. The binding to the PGN may be explained by possible CH-π interactions between aromatic residues (such as Tyr) and the glycan strands of the PG. The interaction may be further stabilized by hydrogen bonds via a water molecule between glycan and Lys. Therefore, both Y60 and K70 may have roles in protein binding to the glycan moiety **(publication 4.3)**. **These results are the first to show the endolysin surface residues, other than the residues present in the PG-binding groove that play a role in substrate binding.** Interesting region of the Ts2631 endolysin was its N-terminal highly positively charged tail with six arginines and one lysine within first 20 residues of the enzyme. The results showed that the N-terminal extension allows endolysin to pass the outer membrane and then to interact with peptidoglycan. Docking of the protein to the PG has been performed by Lukasz Kozlowski, PhD. PG coordinates were provided by Prof. Shahriar Mobashery (University of Notre Dame, USA). The model of Ts2631 endolysin with the PG shows interactions of catalytic core of the enzyme with PG mesh as well as possible conformations of the N-terminal extension, which helps to anchor the protein to the PG (Fig. 2).

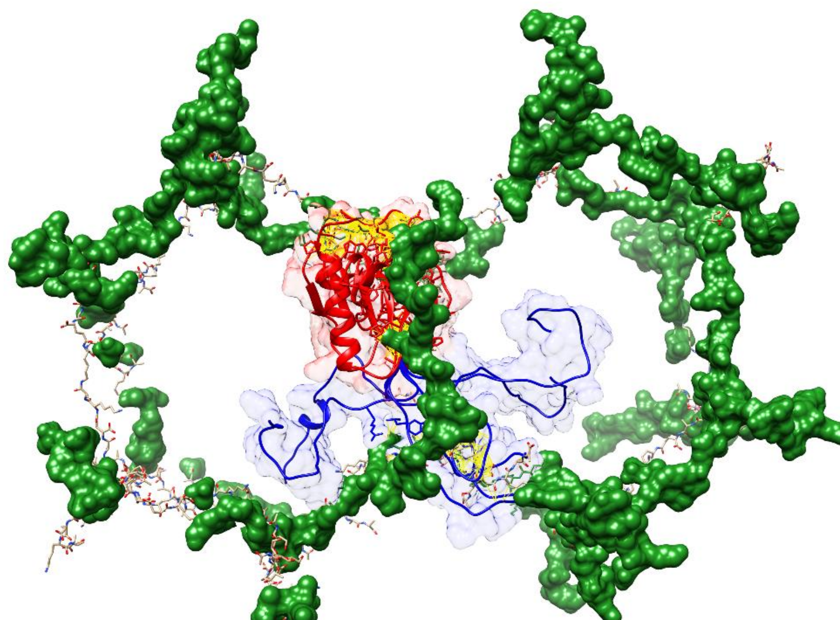

Fig. 2. Model of Ts2631 endolysin binding to peptidoglycan. Shown are the enzyme's globular catalytic core (red) and the flexible N-terminal region (blue), which can adopt a variety of conformations. Both parts of the protein are necessary for peptidoglycan binding (possible interactions are shown in yellow) (publication 4.3).

The obtained results allowed to link the crystal structure data with Ts2631 endolysins functional studies and to make an important contribution to understanding of thermophilic endolysins structure-function relationship.

**D. Ts2631 endolysin from the extremophilic *Thermus scotoductus* bacteriophage vB_Tsc2631 as an antimicrobial agent against Gram-negative multidrug-resistant bacteria.** Viruses 11(7), 657; 2019.

The discovery of antibacterial properties of Ts2631 endolysin against *Thermus thermophilus* HB8 cells without permeabilization of the outer membrane (the intrinsic antibacterial features of endolysins of Gram-negative background are relatively rare) prompted me to pursue this path of research. The main question was <u>if it is possible to use the Ts2631 endolysin as an antibacterial agent?</u>

The application of endolysins in combination with week organic acids or EDTA (cation chelator) facilitated the enzymes to pass an outer membrane and proved to be successful in elimination of Gram-negative bacteria. EDTA, citric and malic acids are natural compounds widely used in food, pharmaceutical or cosmetic industries and there are generally recognized as safe (GRAS) for human use (Oliveira et al., 2014). I showed the increased activity of the Ts2631 endolysin against *Thermus thermophilus* HB8 cells in the presence of 0.5 and 1 mM EDTA and the opposite results for 2 mM citric acid and 5 mM malic acid.

The collaboration between Prof. Tadeusz Kaczorowski and Marek Bronk, PhD (Laboratory of Clinical Microbiology, University Clinical Centre, Gdansk) resulted in the receipt of seven multidrug-resistant (MDR) clinical strains of Gram-negative bacteria with patterns of antibiotic resistance provided for each strain. All strains were deposited by me in the Collection of Plasmids and Microorganisms (KPD) at the University of Gdansk. Two more strains of *Pseudomonas aeruginosa* I have received from the KPD.

The antibacterial tests carried at in the non-optimal temperature for enzyme functionality (37 °C) showed its bactericidal activity against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. **The Ts2631 endolysin is the first from thermophilic bacteriophage with experimentally proven antibacterial activity at 37 °C.** Both, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* belong to the order *Pseudomonadales*. Bacteria of order *Enterobacteriales* such as *Escherichia coli*, *Citrobacter freundii*, *Citrobacter braakii*, *Klebsiella pneumoniae* or *Enterobacter cloacae* were not susceptible to endolysin antibacterial activity. The addition of 0.5 mM EDTA sensitized these bacteria to the action of Ts2631 endolysin. In the presence of EDTA **I have shown the activity of the Ts2631 endolysin against all tested bacterial strains**. In particular, I observed a complete elimination of *C. braakii* (>6 logs). Analogous experiments in the presence of malic and citric acid I have performed with a MSc. student Sebastian Dorawa under my supervision. Similarly to test with use of *T. thermophilus* HB8, the addition of organic acids only slightly improved the activity of the enzyme in case of Gram-negative mesophilic bacteria. The highest log reduction has been achieved in case of *A. baumannii* CRAB KPD 205 (3.30 ± 0.09) in the presence of citric acid (publication 4.4). The collaboration with Magdalena Narajczyk, PhD (Laboratory of Electron Microscopy, Faculty of Biology, University of Gdansk) resulted is transmission electron microscopy (TEM) experiments that showed bactericidal activity of Ts2631 endolysin. With use of TEM and *T. thermophilus* HB8 cells as a substrate it was possible to observe the degradation of peptidoglycan layer of bacterial cell wall. **In case of *A. baumannii* unique photos showed extensive leakage of the cytoplasm through point openings made by the endolysin.** Moreover, I planned the experiments with use of SynapoRed C2 dye, that is specific towards bacterial membranes. The collaborative work with Malgorzata Kapusta, PhD

(Department of Plant Cytology and Embryology, Faculty of Biology, University of Gdansk) resulted in fluorescence microscopy experiments **depicting the dual function of the Ts2631 endolysin involved in both outer membrane destabilization and peptidoglycan degradation.** The fluorescence microscopy experiments showed many visible cell debris after addition of the enzyme to *A. baumannii* cells suspension and the cell membranes of Ts2631 endolysin-treated bacteria were not properly stained. Moreover, images of some cells taken by differential interference contrast microscopy merged with DAPI staining highlighted the proper shape of cells, which was not reflected by SynaptoRed C2 staining. This indicated that the bacterial envelope damage preceded osmotic lysis, which would be expected due to the mode of action of Ts2631 endolysin. The designing, overproduction and purification of the truncated Ts2631 endolysin variant (Δ2-22) allowed to observe the lack of cell lysis in case of the enzyme without the N-terminal region. This result allowed to conclude that the N-terminal part of the enzyme is responsible for its membrane passing ability. **The conducted research is extremely important for understanding of the function of thermophilic bacteriophage endolysins.** Because the Ts2631 endolysin is the first extremophilic endolysin with proved antibacterial activity, the research will pave the way for exploration of thermophilic endolysin as antibacterial agents.

**E. Molecular characterization of a novel lytic enzyme LysC from *Clostridium intestinale* URNW and its antibacterial activity mediated by positively charged N-terminal extension.** Int. J. Mol. Sci. 2020, 21(14), 4894.

Both studied thermophilic endolysins, Ph2119 and Ts2631 are similar to several putative lytic enzymes from bacteria of genus *Clostridium*. After discussion with Anna Kaczorowska, PhD for further analysis I have chosen the putative lytic enzyme LysC derived of Gram-positive bacteria *Clostridium intestinale* URNW (the accession no ERK30183.1). With use of the zymogram methodology (the substrate bacteria are mixed with the separating gel in polyacrylamide electrophoresis SDS-PAGE) I experimentally proved the bacteriolytic activity of LysC enzyme against cells of *C. intestinale* DSM 6191, as well as *C. sporogenes* DSM 767, *B. cereus* ATCC 13061, *M. luteus* ATCC 4698 and *S. aureus* ATCC 25923. The enzyme was not active against *C. perfringens* Cp39 and *B. subtilis* 168 DSM 23778. However, routine turbidity reduction assays (TRA) used in our laboratory to test the endolysins activity were not successful in case of LysC. The optical density of *C. intestinale* DSM 6191 cells suspension didn't decrease after the addition of the enzyme in relation to the control. Therefore, it was impossible to simply test the enzyme working optima in contest of pH, NaCl and temperature. Moreover, the anaerobic bacteria of genus *Clostridium* are difficult to culture and colony forming units are difficult to count in standard antibacterial tests.

Because the LysC enzyme was active against *S. aureus* ATCC 25923 in the zymogram assays I decided to use this bacteria as a substrate in antibacterial tests. More than 5 log reduction in bacterial *S. aureus* cells count after addition of LysC was very promising therefore I decided to continue the studies. **Experimental validation of activity of proteins with annotated lytic function is very important from practical point of view when considering these proteins as antibacterial agents.** Therefore, after comparative analysis **(publication 4.5)** I decided to experimentally validate the functionality of the catalytic centre of the enzyme. In the active site of LysC there are two histidines (H50 and H51), tyrosine (Y76) and cysteine (C155). For site-directed mutagenesis I have chosen also three conserved residues placed near the peptidoglycan binding groove threonine (T52), histidine (H147) and threonine (T153). To

my surprise, variants of LysC with mutated catalytic centre were only slightly less active than the native protein. The results were unexpected as similar experiments with use of Ts2631 endolysin showed a correlation between the presence of conserved residues and enzyme's lytic activity. Therefore, I performed the transmission electron microscopy studies to reveal the mechanism of LysC antibacterial activity. Addition of LysC to *S. aureus* cells led to significant morphological alterations. In many cells the aberrant septum was displayed with attached mesosome-like structures, suggesting abnormal cell division. This picture was clearly different than that observed for the Ts2631 endolysin were peptidoglycan degradation was observed. The question raised, what properties of the LysC enzyme are responsible for its antimicrobial activity? Similarly to Ts2631 endolysin, LysC also have N-terminal highly positively charged region. The analysis of the crystal structure of the LysC protein performed in the framework of this work showed that this region is intrinsically disordered and belongs to the so-called intrinsically disordered regions (IDRs). *In silico* analyses using the AMPA web application to assess the potential presence of antimicrobial domains in proteins and the APD3 antimicrobial peptide calculator revealed that this region may act as an antimicrobial peptide through interactions with bacterial membranes. To verify this hypothesis, I performed site-directed mutagenesis to remove the N-terminal region of LysC. The truncated variant (LysC Δ2-23) turned out to be inactive against *S. aureus* cells, suggesting involvement of the deleted region in the antibacterial activity of the enzyme. Next, as a result of the collaboration with Prof. Elzbieta Jankowska (Department of Biomedical Chemistry, Faculty of Chemistry, University of Gdansk) I received the synthetic peptide corresponding to the first 30 residues of the LysC protein with 11 positively charged and 10 hydrophobic residues. The peptide turned out to be active against *S. aureus* ATCC 25923 at a level corresponding to that of the recombinant enzyme. Next, MSc. Monika Szadkowska during her PhD project, used circular dichroism spectroscopy and investigated the secondary structure of the peptide without and in the presence of detergents that mimic bacterial membranes. The results show that the peptide in the presence of sodium dodecyl sulfate (SDS) and n-dodecylphosphocholine (DPC) adopts α-helical conformation. Many α-helical antibacterial peptides are unstructured in solution, but in the presence of membranes they adopt an amphipathic helical structures. This research was partially financed by the National Science Center within the MINIATURA3 project.

**In the present work for the first time I experimentally validated the antibacterial activity of the lytic enzyme LysC from *C. intestinale* URNW. I revealed the mechanism of its antibacterial activity mediated by N-terminal highly positively charged region. Moreover, I characterized novel synthetic peptide called Intestinalin that corresponded to the first 30 residues of LysC protein.**

**My most important scientific achievements are:**

- Experimental validation of lytic activity of two putative bacteriophage endolysins Ph2119 and Ts2631, as representatives of very small group of so far unexplored endolysins from thermophilic bacteriophages, and the analogous protein LysC derived from bacteria *Clostridium intestinale* URNW.
- Experimental validation of the catalytic site of the Ts2631 endolysin as a first representative of extremophilic endolysins.

- Determination of thermal stability of endolysins Ph2119 and Ts2631, including determination of melting temperature (Tm) of endolysin Ts2631 as the first representatives of endolysins from thermophilic bacteriophages.
- The functional and structural characteristics of Ts2631 endolysin, including the discovery of residues other than the residues present in the PGN-binding groove that play a role in the substrate binding.
- The discovery of the antibacterial potential of the Ts2631 endolysin to kill Gram-negative multidrug-resistant bacterial pathogens.
- Determination of the membrane destabilization activity of the highly positively charged N-terminal regions of proteins with lytic function (based on the Ts2631 endolysin (Fig. 3) and the lytic protein LysC), what may help to understand the function of similar regions of other antimicrobial proteins.
- Characterization of the antibacterial function of novel, synthetic peptide Intestinalin.

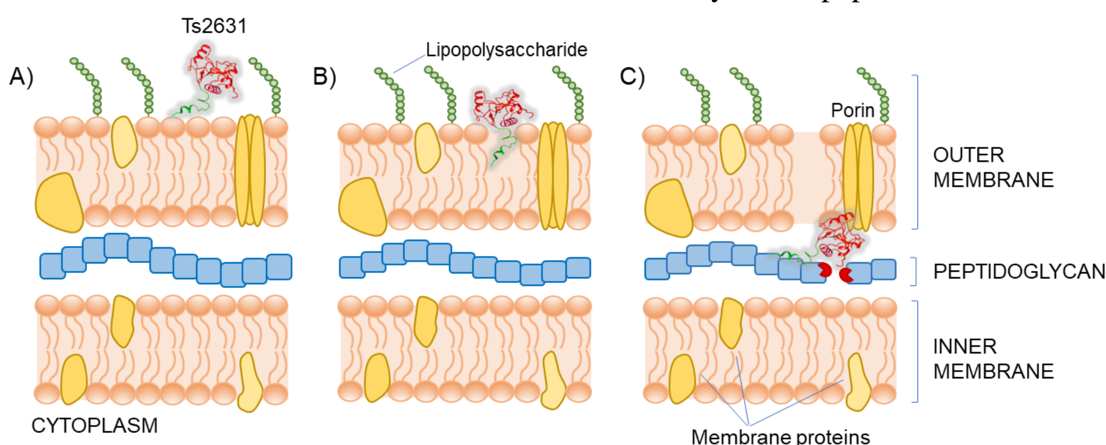


Fig. 3. Antibacterial activity of the Ts2631 endolysin. Through the positively charged N-terminal region, the protein interacts (A) and destabilizes (B) the bacterial outer membrane, then degrades the peptidoglycan layer (C) via catalytic activity (the protein has an N-acetyluramoyl-L-alanine amidase domain).

In 2014, Prof. Rob Lavigne and Prof. Yves Briers introduced the term Artilysin® to describe recombinant phage enzymes with ability to penetrate the outer membrane of Gram-negative bacteria. Special approach has been then developed to engineer enzymes combining a polycationic nonapeptide PCNP with modular endolysins (Briers et al., 2014). Moreover, in 2020 a high-throughput hit-to-lead development platform for engineered lysins has been proposed (called a VersaTile-driven platform) to construct thousands of endolysin variants with different substrate specificities, stability or efficacy (Gerstmans et al., 2020). This VersaTile-driven platform could offer new opportunities to deliver novel engineered lysins, consisting of different combinations of outer membrane penetrating peptides (OMPs), linkers, CBDs, and EADs for diverse, also therapeutic applications.

Within presented work I characterized novel, globular, thermostable endolysins that may serve as building blocks to generate engineered endolysins with higher thermal stability. Moreover, the synthetic peptide Intestinalin similarly to PCNP, can be used as a polycationic peptide for use in fusion with various endolysins to improve their lytic activity. Primary sequence of Intestinalin corresponds to first 30 residues of LysC lytic enzyme isolated from

*Clostridium intestinale* URNW, therefore in my work I found a natural counterpart of engineered Artilysins.

**5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions.**

**DESCRIPTION OF SCIENTIFIC WORK**

I began my Master's studies in 1995 at the Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk. Already during the second year of studies, I started the laboratory work in a group of Prof. Janusz Limon (Department of Biology and Genetics, Medical University of Gdansk). The work resulted in first congress reports (under my maiden name Szczygiel):

- M. Perkowska, **M. Szczygiel**: Influence of sodium nitroprusside (NaNP) on sister chromatid exchange frequency and cell kinetics in cultured human lymphocytes. May 1998, 6-th International Students' scientific Conference, Gdansk, Poland
- M. Perkowska, **M. Szczygiel**: Influence of diethylenetriamnine (DETA) and sodium nitroprusside (NaNP) on sister chromatid exchange frequency and cell kinetics in cultured human lymphocytes. 22-25$^{th}$ of September 1998, 13$^{th}$ Symposium of Polish Society of Genetics, Warsaw, Poland
- **M. Szczygiel**, M. Gruchala, W. Dubaniewicz, K. Ochman, D. Ciecwierz, A. Rynkiewicz, J. Limon: The PLA1/PLA2 glycoprotein IIIa Polymorphism is not associated with increased risk for coronary artery disease and myocardial infraction in the population of the north region of Poland. 24-26$^{th}$ of May 1999, 2$^{nd}$ Symposium of Polish Society of Human Genetics, Poznan, Poland

and in 2001 my first publication:

- M. Perkowska, **M. Szczygiel,** A. Wozniak, J. Limon: Influence of diethylenetriamnine (DETA) and sodium nitroprusside (NaNP) on sister chromatid exchange frequency and cell kinetics in cultured human lymphocytes. J. Appl. Genet. 42(1), 2001, p. 233-235

In February 1998 I also accomplished my first research visit to the Institute of Mother and Child, Warsaw, Poland in a group of Prof. Jerzy Bal (Medical Genetics Department). Moreover, as a 4th year student I participated in the organization of the 7th Symposium of the European Society for the Study of Purine and Pyrimidine Metabolism in Man, Gdansk, Poland (15-19$^{th}$ September 1999) taking part in the organization of the sightseeing tours and registration of participants.

I graduated in September 1999 and a month later I started further work at the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Medical University of Gdansk. Under a supervision of Wladyslaw Werel, PhD, I worked on regulation of the transcription initiation in bacteria, especially the regulatory roles of sigma factors. There I gained experience in working with DNA (gene cloning) and in proteins purification techniques using mostly High Performance Liquid Chromatography (HPLC). At the Department of Pharmaceutical Microbiology I started to work as an academic teacher conducting practical courses of Microbiology for students of the Faculty of Pharmacy.

In 2002, I started a 15-month research internship in the team of Prof. Lea Sistonen, Åbo Akademi University, Turku Center for Biotechnology, Turku, Finland, receiving a CIMO (Center for International Mobility) scholarship under the patronage of the Finnish Ministry of Education and Culture. In the laboratory of Prof. Sistonen I studied the cellular stress responses and the regulation of mammalian heat shock factors (HSFs). The research interest of the group was mainly focused on the biology of HSF1 and HSF2.

After returning to Poland in 2003, I became a student of the Doctoral Studies at the parent Intercollegiate Faculty of Biotechnology of the University of Gdansk and the Medical University of Gdansk. In the team of Prof. Jarosław Marszalek, I conducted research on the role of the mitochondrial chaperone Hsp40 (Mdj1) and the bifunctional Ilv5 protein (the main role in the biosynthesis of branched chain amino acids) in maintaining the mitochondrial DNA of the yeast *Saccharomyces cerevisiae*. The results of conducted research were published in the form of two publications in the highly scored journal Biochimica et Biophysica Acta - Molecular Cell Research (MNiSW2020 points = 140; **Annex 4.4, point 11 and 12**):

- M. Macierzanka*, **M. Plotka*,** D. Pryputniewicz-Drobinska, A. Lewandowska, R. Lightowlers, J. Marszalek: Maintenance and stabilization of mtDNA can be facilitated by the DNA-binding activity of Ilv5p. Biochim Biophys Acta. 1783(1), 2008, p.107-117. *equal contribution; ($IF_{2008}$ 4.893; $MNiSW_{2015}$ = 40).

- G. L. Ciesielski*, **M. Plotka*,** M. Manicki*, B. A. Schilke, R. Dutkiewicz, Ch. Sahi, J. Marszalek, E. A. Craig: Nucleoid localization of Hsp40 Mdj1 is important for its function in maintenance of mitochondrial DNA. Biochim Biophys Acta. 1833(10), 2013, p. 2233-2243. * equal conribution; ($IF_{2013}$ 5.297; $MNiSW_{2015}$ = 40)

The defense of the PhD thesis entitled 'Protein components of the mitochondrial nucleoid of the yeast *Saccharomyces cerevisiae*' was held in December 2007. The reviewers were: Prof. Michal Obuchowski from the Intercollegiate Faculty of Biotechnology, UG and MUG (University of Gdansk and Medical University of Gdansk) and Prof. Hanna Kmita from the Adam Mickiewicz University in Poznan. After submitting my doctoral dissertation, in September 2007, I started a postdoctoral research internship at the Department of Gynecology and Obstetrics, John Radcliffe Hospital, Oxford University, UK. In the team of Prof. Joanna Poulton, I investigated the relationship between the occurrence of the mitochondrial DNA variant (called the OriB or the 16189 variant; T→C transition at position 16189 of mtDNA) and the risk of type 2 diabetes in humans (publication **Annex 4. 4, point 10**):

- Z. Ye, Ch. Gillson, M. Sims, K-T. Khaw, **M. Plotka**, J. Poulton, C. Langenberg, N. J. Wareham: The association of the mitochondrial DNA OriB variant (16184-16193 polycytosine tract) with type 2 diabetes in Europid populations. Diabetologia 56(9), 2013, p. 1907-1913. ($IF_{2013}$ = 6.880; $MNiSW_{2015}$ = 40)

I returned to Poland at the beginning of 2010, and then started to work as an assistant at the Laboratory of Molecular Biology and Cytogentics, Invicta Diagnostic Medical Laboratories at the Professor Hilary Koprowski Gdansk Science and Technology Park (GSTP). In the laboratory, in cooperation with the scientific community of the University of Gdansk, I implemented diagnosis of HCV virus causing hepatitis C and molecular diagnostics of borreliosis (Lyme disease) caused by *Borrelia burgdorferi*. In the academic year 2011/2012 I also conducted practical course of Microbiology with students of the Faculty of Pharmacy, Medical University of Gdansk.

In 2012, I begun to work as a senior specialist at the Department of Microbiology at a parental University of Gdansk within a project 'Exgenome Molecular Enzymes' (EXGENOMES) granted under the European Union's 7th Framework Program. The main objective of the EXGENOMES project was to develop new and improved thermostable enzymes mainly for use, as reagents, in large-scale DNA synthesis. The target source for the new enzymes were self-replicating mobile genetic elements (especially bacteriophages) from thermophilic bacteria isolated from water of Icelandic hot springs. The groups of enzymes of research interest were polymerases (DNA and RNA), ligases, nucleases, reverse transcriptases, polynucleotide kinases, lysozymes and more. Mentioned groups of proteins were also of special interest of two biotechnology companies engaged in the project Prokazyme (Iceland) and A&A Biotechnology (Poland). As a team member I focused on the characterization of potential lysozymes.

When I joined the project, I was already an experienced researcher with wide methodological background. At the Department of Microbiology, I optimized and implemented a number of methods that allow me to study the lytic activity of proteins. At that time, the only one characterized lysozyme isolated from extremophile was the enzyme φIN93 derived from the bacteriophage of *Thermus aquaticus* TZ2 (Matsushita & Yanase 2008). However, the φIN93 enzyme was purified only in a small quantity (0.0072 mg / 600 ml of bacterial lysate) so it was difficult to achieve an amount sufficient to fully investigate its lytic activity. The φIN93 enzyme was very specific lysing only bacteria from *Thermus* order, leaving the mesophilic bacteria untouched. Therefore, the enzyme was not attractive in terms of application as an antibacterial agent. During my work within the EXGENOMES project (and also within the next project called Virus-X) I characterized two thermostable lytic enzymes (endolysins) derived from *Thermus scotoductus* bacteriophages. The obtained results made the substantial contribution to understanding of the functions of these class of enzymes isolated from extremophilic environments. Two enzymes showed broad substrate specificity for peptidoglycan of both thermophilic and mesophilic bacteria, the latest represented by *Escherichia coli* or *Pseudomonas fluorescens*. The enzymes did not lyse Gram-positive bacteria such as *Lactococcus lactis* or *Staphylococcus aureus*. However, the biotechnological companies involved in the project were moderately interested in lytic enzymes specific for Gram-negative bacteria. Therefore, I continued the study asking specific research questions.

- **How the investigated enzymes bind peptidoglycan layer of bacterial cell wall without the CBD domain?**
- **Is it possible to apply these enzymes as antibacterial agents to treat bacterial infections at 37 °C, 23 °C below their optimal operating temperature?**
- **What are the features of other lytic enzymes derived from mesophilic bacteria that have spatial architecture similar to investigated thermophilic endolysins?**

Identification of amino acids essential for peptidoglycan binding by the thermostable Ts2631 endolysin, determination of the role of the N-terminal region of Ts2631 enzyme in substrate recognition **(publication 4.3)**, or confirmation of the antibacterial activity of Ts2631 endolysin at 37 °C against bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* **(publication 4.4)** are the answers to the questions asked. Moreover, I characterized the activity of LysC lytic enzyme derived from *C. intestinale* URNW that shows 34% primary sequence identity to Ph2119 endolysin and 33% to Ts2631 (**publication 4.5**).

Among proteins showing similarity to the thermostable endolysins Ph2119 and Ts2631 there is a potential N-acetylmuramoyl-L-alanine amidase, LysB derived from the prophage of *Clostridium botulinum*. The lytic activity of the enzyme has been experimentally confirmed within a project conducted together with a PhD student, Agnieszka Morzywolek. Results of

performed experiments are included in the manuscript: 'Newly identified lytic enzyme from prophage of *C. botulinum* E3 strain Alaska E43, which shows activity against cells of *Clostridium* genus', which is in the final stage of preparation.

## ESTABLISHED COLLABORATIONS

- Danuta Augustin-Nowacka, PhD (Physics-Chemistry Workshops, Faculty of Chemistry, University of Gdansk) – Circular Dichroism (CD) spectroscopy measurements

- Prof. Elzbieta Jankowska, (Department of Biomedical Chemistry, Faculty of Chemistry, University of Gdansk) – synthesis of antibacterial peptides

- Malgorzata Kapusta, PhD (Department of Plant Cytology and Embryology, Faculty of Biology, University of Gdansk) – fluorescence microscopy experiments

- Prof. Joanna Makowska (Department of General and Inorganic Chemistry, Faculty of Chemistry, University of Gdansk) – Differential Scanning Calorimetry (DSC) analysis

- Prof. Shahriar Mobashery (University of Notre Dame, USA) – peptidoglycan coordinates

- Prof. Magdalena Narajczyk (Laboratory of Electron Microscopy, Faculty of Biology, University of Gdansk) – transmission electron microscopy

- Steven M. Swift, PhD (USDA ARS NEA BARC Animal Biosciences and Biotechnology Laboratory, Beltsville, USA) – analysis of activity of lytic enzymes against *Clostridium perfringens* cells

- Roman Szczepanowski (the Core Facility, International Institute of Molecular and Cell Biology in Warsaw) – analytical ultracentrifugation

In addition, as part of two European Union projects, I have established cooperation with scientists from many research centers and biotechnology companies. These centers are listed in Annex 4, point 14 (List of scientific or artistic achievements which present a major contribution to the development of a specific discipline).

At this point, I would like to emphasize the important role of Prof. Tadeusz Kaczorowski in obtaining EU funds for conducting research under two international projects: EXGENOMES and Virus-X (Appendix 4, point 14). Working in these projects allowed me to meet and gain knowledge from world-class experts in the field of gene cloning, enzyme purification and characterization, or protein crystallography. As a result of the joined effort three novel enzyme's structures were deposited in the PDB database (this appendix, point 6.C). Moreover, the results of the team work was also a publication aiming in characterizing the XepA and YomS proteins from the PBSX prophage of *Bacillus subtilis* 168 lytic cassette. The work has been published in Acta Crystallographica section D with $IF_{2019} = 5.266$ (Appendix 4, point 4.2).

- A. Stefanska, A. K. Kaczorowska, **M. Plotka**, O. H. Fridjonsson, G. O. Hreggvidsson, S. Hjorleifsdottir, J. K. Kristjansson, S. Dabrowski, T. Kaczorowski: Discovery and characterization of RecA protein of thermophilic bacterium *Thermus thermophilus* MAT72 phage Tt72 that increases specificity of a PCR-based DNA amplification. J Biotechnol 182-183, 2014, p. 1-10. ($IF_{2015}$ 2.871; $MNiSW_{2015} = 40$).

- A. Stefanska, L. Gaffke, A. K. Kaczorowska, **M. Plotka**, S. Dabrowski, T. Kaczorowski: Highly thermostable RadA protein from the archaeon Pyrococcus woesei enhances specificity of simplex and multiplex PCR assays. J Appl Genet 57, 2016, p. 239-249. ($IF_{2016} = 1.655$; $MNiSW_{2019} = 100$)

- S. Freitag-Pohl, A. Jasilionis, M. Håkansson, L.A. Svensson, R. Kovačič, M. Welin, H. Watzlawick, L. Wang, J. Altenbuchner, **M. Płotka**, A-K. Kaczorowska, T. Kaczorowski, E. Nordberg, S. Al-Karadaghi, B. Walse, A. Aevarssong, E. Pohl,: Crystal structures of the *Bacillus subtilis* prophage lytic cassette proteins XepA and YomS. Acta Crystallographica section D 75(11), 2019, p. 1028-1039; https://doi.org/10.1107/S2059798319013330 ($IF_{2019}$ = 5.266; $MNiSW_{2019}$ = 100).

## RESEARCH PLANS

Working within these projects (EXGENOMES and Virus-X) helped me to identify and to develop my own research interests regarding the practical application of lytic enzymes isolated from bacteria and bacteriophages. That led to cooperation with Prof. Yves Briers (Department of Biotechnology, Faculty of Bioscience Engineering, Ghent University, Belgium). The collaboration aimed in creation, production and characterization of recombinant fusion proteins with improved antibacterial activity, part of which would be the domains of studied by me endolysins. Joint research started in 2017 (e.g. as part of my month-long research internship in Ghent, Belgium) and yielded in promising preliminary results. That will be the starting point for writing the OPUS grant application, which I hope to submit by December 15, 2020 in the 20th edition of the proposal.

Moreover, the results showing that the N-terminal region of LysC lytic enzyme is responsible for its antibacterial activity prompted me to investigate the antibacterial potential of the peptide Intestinalin synthesized on the basis of first 30 amino acids of the enzyme. The detailed work on antibacterial properties of the peptide as well as its spatial architecture are currently conducted within MINIATURE 3 project granted to me by National Polish Centre (NCN), decision number 2019/03/X/NZ1/00394. The title of the project is '*Analysis of the structure and function of synthetic peptides derived from N-terminal regions of lytic enzymes with particular emphasis on characterization of novel antibacterial peptide Intestinalin'*. The results of the project were partially published in one of the publications of the scientific achievement (publication 4.5). The analysis of the spatial structure of Intestinalin with the use of nuclear magnetic resonance spectroscopy (NMR), are already completed and will be included in the next publication.

Another interesting question is whether similar, highly positively charged regions of other potential lytic proteins also play a role in their lytic function? The preliminary results were achieved within MINIATURE 3 project. Five potential lytic enzymes with N-terminal regions similar to N-terminal part of the LysC were purified and partially characterized and I would like to continue this research also in the future.

The next point is **to explore the thermostability phenomenon of investigated enzymes**. It is known that in terms of the structure thermostable enzymes resemble their mesophilic counterparts. The examples include thermostable Ts2631 endolysin and mesophilic T7 lysozyme **(publication 4.3, Figure 4)**. However, factor that might contribute to the stabilization of Ts2631 endolysin is the formation of extended hydrophobic core by the high number of tryptophans (4.49%). In mesophiles, the percentage of tryptophans on average does not exceed 1.27% (Kozlowski, 2017). Tryptophan residues are known to participate in cation-π interactions that maintain the conformational stability of protein structures (Dougherty, 1996; Pack & Yoo, 2004). The stabilizing effect is also caused by an increased amount of proline or arginine (the latter can stabilize the structure of proteins through interactions with ions).

It is extremely interesting whether the presence of any of these amino acids has a significant impact on the thermostability of the tested Ts2631 and Ph2119 endolysins? If so,

will substitution of corresponding residues in mesophilic proteins increase their thermal stability? I will try to answer these questions in my further research.

## SUMMARY

Summarizing, my achievements include 14 articles published in journals from the JCR list, ten of which were written after I obtained my PhD degree. Five articles constitute the achievement presented for evaluation. I am the first author of eight publications (two on the basis of an equal contribution), and a corresponding author of three of them. The total impact factor of all my publications is 48.5, while the number of citations is 125 (according to the Scopus database), the Hirsch index is 8. The total score according to the list A of the MNiSW (the Annex to the Communication of the Minister of Science and Higher Education of December 18, 2019) is 1510, including five publications for 140 points.

I have participated in three international research projects, two of which were multi-center grants financed under European Union programs. My latest research results were honored in 2020 with the team award of the 3rd degree of the Rector of the University of Gdansk for the achievement: *Molecular characterization of proteins with biotechnological potential on the example of unique restriction endonucleases and phage endolysins*. I presented the results of my work at 31 conferences, including 8 oral presentations (3 as an invited speaker) and I was the Advisory Board Member of two of them (Bacteriophage 2016 and Bacteriophage 2017 taking place in London, UK). I reviewed both original papers and reviews for international journals from the JRC list (14 reviews). Moreover, I take an active part in the life of the Faculty of Biology, University of Gdansk e. g. as a member of the Program Committee of the course Genetics and Experimental Biology, or by giving lectures for students of Pomeranian high schools as part of the projects "Invite a scientist to school" or "InnovaBio Pomorze" under the patronage of the Pomeranian Science and Technology Park in Gdynia.

## SUPPLEMENTARY LITERATURE

Bastos MD, Coutinho BG, and Coelho ML. 2010. Lysostaphin: A Staphylococcal Bacteriolysin with Potential Clinical Applications. *Pharmaceuticals (Basel)* 3:1139-1161. 10.3390/ph3041139

Borysowski J, Weber-Dabrowska B, and Górski A. 2006. Bacteriophage endolysins as a novel class of antibacterial agents. *Exp Biol Med (Maywood)* 231:366-377.

Briers Y, Lavigne R, Hertveldt K, Hanssens I, Engelborghs Y, and Volckaert G. 2007. Stability of phiKMV lysin gp36c reflects its role during bacteriophage infection. *Commun Agric Appl Biol Sci* 72:115-118.

Briers Y, Walmagh M, Van Puyenbroeck V, Cornelissen A, Cenens W, Aertsen A, Oliveira H, Azeredo J, Verween G, Pirnay JP, Miller S, Volckaert G, and Lavigne R. 2014. Engineered endolysin-based "Artilysins" to combat multidrug-resistant gram-negative pathogens. MBio 5:e01379-01314. 10.1128/mBio.01379-14

Cheng X, Zhang X, Pflugrath JW, and Studier FW. 1994. The structure of bacteriophage T7 lysozyme, a zinc amidase and an inhibitor of T7 RNA polymerase. *Proc Natl Acad Sci U S A* 91:4034-4038.

Desmarais SM, Cava F, de Pedro MA, and Huang KC. 2014. Isolation and preparation of bacterial cell walls for compositional analysis by ultra performance liquid chromatography. *J Vis Exp*:e51183. 10.3791/51183

Dougherty DA. 1996. Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. Science 271:163-168.

Frickey T, and Lupas A. 2004. CLANS: a Java application for visualizing protein families based on pairwise similarity. Bioinformatics 20:3702-3704. 10.1093/bioinformatics/bth444

Gerstmans H, Grimon D, Gutiérrez D, Lood C, Rodríguez A, van Noort V, Lammertyn J, Lavigne R, and Briers Y. 2020. A VersaTile-driven platform for rapid hit-to-lead development of engineered lysins. *Sci Adv* 6:eaaz1136. 10.1126/sciadv.aaz1136

Gutiérrez D and Briers Y. 2021. Lysins breaking down the walls of Gram-negative bacteria, no longer a no-go. *Current Opinion in Biotechnology* 68:15–22. 10.1016/j.copbio.2020.08.014

Kozlowski LP. 2017. Proteome-pI: proteome isoelectric point database. Nucleic Acids Res 45:D1112-D1116. 10.1093/nar/gkw978

Kumar S, Tsai CJ, and Nussinov R. 2000. Factors enhancing protein thermostability. *Protein Eng* 13:179-191. 10.1093/protein/13.3.179

Lewis K. 2012. Antibiotics: Recover the lost art of drug discovery. *Nature* 485:439-440. 10.1038/485439a

Love MJ, Bhandari D, Dobson RCJ, and Billington C. 2018. Potential for Bacteriophage Endolysins to Supplement or Replace Antibiotics in Food Production and Clinical Care. Antibiotics (Basel) 7. 10.3390/antibiotics7010017

Matsushita I, and Yanase H. 2008. A novel thermophilic lysozyme from bacteriophage phiIN93. *Biochem Biophys Res Commun* 377:89-92. 10.1016/j.bbrc.2008.09.101

Nelson D, Loomis L, and Fischetti VA. 2001. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proc Natl Acad Sci U S A* 98:4107-4112. 10.1073/pnas.061038398

Oliveira H, Melo LD, Santos SB, Nóbrega FL, Ferreira EC, Cerca N, Azeredo J, and Kluskens LD. 2013. Molecular aspects and comparative genomics of bacteriophage endolysins. *J Virol* 87:4558-4570. 10.1128/JVI.03277-12

Oliveira H, São-José C, and Azeredo J. 2018. Phage-Derived Peptidoglycan Degrading Enzymes: Challenges and Future Prospects for In Vivo Therapy. *Viruses* 10. 10.3390/v10060292

Oliveira H, Thiagarajan V, Walmagh M, Sillankorva S, Lavigne R, Neves-Petersen MT, Kluskens LD, and Azeredo J. 2014. A thermostable *Salmonella* phage endolysin, Lys68, with broad bactericidal properties against gram-negative pathogens in presence of weak acids. *PLoS One* 9:e108376. 10.1371/journal.pone.0108376

O' Neill J. 2016. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations (The Review on Antimicrobial Resistance). https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf

Pack SP, and Yoo YJ. 2004. Protein thermostability: structure-based difference of amino acid between thermophilic and mesophilic proteins. J Biotechnol 111:269-277. 10.1016/j.jbiotec.2004.01.018

Peng SY, You RI, Lai MJ, Lin NT, Chen LK, and Chang KC. 2017. Highly potent antimicrobial modified peptides derived from the *Acinetobacter baumannii* phage endolysin LysAB2. Sci Rep 7:11477. 10.1038/s41598-017-11832-7

Schmelcher M, Donovan DM, and Loessner MJ. 2012. Bacteriophage endolysins as novel antimicrobials. *Future Microbiol* 7:1147-1171. 10.2217/fmb.12.97

Thandar M, Lood R, Winer BY, Deutsch DR, Euler CW, and Fischetti VA. 2016. Novel Engineered Peptides of a Phage Lysin as Effective Antimicrobials against Multidrug-Resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother 60:2671-2679. 10.1128/AAC.02972-15

Vermassen A, Leroy S, Talon R, Provot C, Popowska M, and Desvaux M. 2019. Cell Wall Hydrolases in Bacteria: Insight on the Diversity of Cell Wall Amidases, Glycosidases and Peptidases Toward Peptidoglycan. *Front Microbiol* 10:331. 10.3389/fmicb.2019.00331

Vollmer W, Blanot D, and de Pedro MA. 2008a. Peptidoglycan structure and architecture. *FEMS Microbiol Rev* 32:149-167. 10.1111/j.1574-6976.2007.00094.x

Vollmer W, Joris B, Charlier P, and Foster S. 2008b. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* 32:259-286. 10.1111/j.1574-6976.2007.00099.x

Ye T, and Zhang X. 2008. Characterization of a lysin from deep-sea thermophilic bacteriophage GVE2. *Appl Microbiol Biotechnol* 78:635-641. 10.1007/s00253-008-1353-1

Yoshida H, Kinoshita K, and Ashida M. 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *J Biol Chem* 271:13854-13860.

Young R. 2014. Phage lysis: three steps, three choices, one outcome. *J Microbiol* 52:243-258. 10.1007/s12275-014-4087-z

## 6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art

### A. Teaching achievements and contributions regarding dissemination of science

• Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Medical University of Gdansk (1999-2002; 2012)
Number of teaching hours per year = 240 hours (1999-2002) and = 65 hours (2012) including:

-Microbiology - practical course for students of Faculty of Pharmacy, 3rd year

• Department of Molecular and Cellular Biology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk (2003-2007)
Number of teaching hours per year as part of the Doctoral Study = 90 hours, including:

- Genetic engineering for students of Faculty of Biotechnology, 1st degree, 2nd year

• Department of Microbiology, Faculty of Biology, University of Gdansk (2014-present)
Number of teaching hours per year = 240, including:

- Microbiology – practical courses for students of Faculty of Biology (Biology, 1st degree 2nd year and Medical biology, 1st degree, 1st year)
- Elements of bacterial genetics – practical course for students of Faculty of Biology (Biology, 1st degree, 3rd year)
- Specialization in microbiology – practical course for students of Faculty of Biology (Biology, 2nd degree, 2nd year)
- Diploma workshop for students of Faculty of Biology (Biology and Medical biology, 2nd degree, 2nd year)
- Seminar for students of Faculty of Biology (Biology, 2nd degree, 2nd year)
- Research at the faculty - lecture classes for students of Faculty of Biology (Biology, 1st degree, 3rd year)
- Project Based Learning (PBL) workshop for students of Faculty of Biology (Biology, 1st degree, 3rd year)

Moreover:

- Member of the Program Committee of the course Genetics and Experimental Biology, Faculty of Biology, 2020 - present;
- Lecture: Alternatives to antibiotics: "How to treat bacterial infections?" for secondary schools in the Pomeranian Voivodeship as part of meetings with scientists; 'InnovaBio Pomorze' project under the patronage of the Pomeranian Science and Technology Park, Gdynia, September 2020.
- Participation in the committee conducting the final examination for students of Faculty of Biology (students of the 3rd year of Bachelor's course) in the years 2016-2020
- Participation in the promotion of the Faculty of Biology; during 'European Antibiotic Awareness Day (EAAD) – conducting workshop, November 2015 or 'Invite a scientist to school' – giving three lectures entitled: "What are the alternatives to antibiotics for

fighting bacterial infections?" for secondary schools in the Pomeranian Voivodeship; years 2019-2020.

- Chairman of the seminar series at the Department of Gynecology and Obstetrics, John Radcliffe Hospital, University of Oxford, Oxford, UK (app. 10 meetings).

## B. Scientific supervision of students

1. As a co-advisor of Master's students:

- Pharmacy, Faculty of Pharmacy, Medical University of Gdańsk = 1 student
- Biotechnology, Intercollegiate Faculty of Biotechnology UG&MUG = 3 students
- Biology and Medical Biology, Faculty of Biology, University of Gdansk = 2 students

2. As a formal supervisor of Bachelor and Master Thesis dissertations:

- Biology and Medical Biology, Faculty of Biology, University of Gdansk;

- a formal supervisor of Master Thesis (2016-2020) = 6 students
- a formal supervisor of Bachelor Thesis = 9 students

3. As a co-advisor of PhD students:

- co-advisor of a PhD student Agnieszka Morzywołek; open doctoral dissertation 16.10.2015; the title of the PhD thesis: 'Characteristics of the lytic enzymes from bacteria of order *Clostridium* showing similarity to eukaryotic peptidoglycan recognition proteins'
- co-advisor of a PhD student Monika Szadkowska; doctoral dissertation not opened

4. As a reviewer of Bachelor and Master Thesis:

- Biology and Medical Biology, Faculty of Biology, University of Gdansk;

- reviews of Bachelor Thesis = 7 students
- reviews of Master Thesis = 2 students

5. Scientific supervision of research projects carried out at the Faculty of Biology of the University of Gdansk

- two practical project within International Baccalaureate (IB) Diploma Programme; Julia Lamparska, Konrad Drozdzewski, Liceum III, Gdansk, 2015
- educational project –students of junior high school no. 13, Gdynia, 2015
- Scientific supervision of ERASMUS student Ivana Charuosova from Slovak University of Agriculture in Nitra – Faculty of biotechnology and food sciences, Slovakia; title of the project: Development of a simple protocol to effectively isolate plasmid DNA from *Thermus flavus* and *Flavobacterium okeanokoites* for use in the downstream applications including automated sequencing", in 2013.

6. Awards for teaching achievement:

- Tutor of awarded Master thesis of Sebastian Dorawa. The title of the thesis: 'Antibacterial activity of Ts2631 endolysin from bacteriophage vB_Tsc2631 and analysis of residues responsible for substrate binding' – 26$^{th}$ of September 2017

## C. Other achievements, not listed in sections A and B

- Depositing the DNA sequences in GenBank NCBI:
  **GenBank: KF408298.1**
  *Thermus* phage 2119 lysozyme gene, complete cds
  **GenBank: KJ561354**
  *Thermus* phage 2631 LysT endolysin gene, complete cds

- Participation in depositing of crystal structures of enzymes:
  **PDB: 6FHG**; Crystal structure of the Ts2631 endolysin from *Thermus scotoductus* phage with the unique N-terminal moiety responsible for peptidoglycan anchoring
  **PDB: 6SU5**; N-acetylmuramoyl-L-alanine amidase Ph2119 from bacteriophage MAT2119
  **PDB: 6SSC**; N-acetylmuramoyl-L-alanine amidase LysC from *Clostridium intestinale* URNW

- Participation in commercialization of one of investigated lytic enzymes (Prokazyme Company, Iceland):
  **ThermoPhageTM Lysozyme** (Product number: Lys164)

- Depositing in the Collection of Plasmids and Microorganisms (KPD), Faculty of Biology, University of Gdansk of 44 bacterial strains including 8 clinical strains of Gram-negative bacteria:
  KPD 205 *Acinetobacter baumannii* CRAB (ang. carbapenem-resistant)
  KPD 217 *Escherichia coli* MBL (+) (ang. metallo-beta-lactamase producing)
  KPD 218 *Citrobacter braakii* MBL (+)
  KPD 219 *Citrobacter freundii* MBL (+)
  KPD 297 *Enterobacter cloacae* MBL (+)
  KPD 298 *Klebsiella pneumoniae* KPC (+) (ang. carbapenemase producing)

## D. Activity towards improving professional qualifications

Pedagogical qualifications:

1. Completed Pedagogical Study (4 semesters) in vocational education at the Gdańsk University of Technology with a very good result (2001)

Participation in Symposia and courses:

1. Bioinnovation International Summit, the Pomeranian Science and Technology Park in Gdynia (2011)
2. Medical University of Gdansk; Course: Project management, International Project Management Association for Intercollegiate Faculty of Biotechnology UG&MUG (2006)
3. 11th Advanced Course on Digital Microscopy and Fluorescence Techniques in Cell Biology, Heidelberg, Germany (2006)
4. TuBS Symposium on Genetic Engineering of Mice for Biology and Disease Models, BioCity, Turku, Finland (2002)

5. The12th Annual BioCity Symposium. Recent breakthroughs in drug development, BioCity, Turku, Finland (2002)

## E. Other publications

1. Plotka M.: From bacteriophages to endolysins: a look at novel antibacterial agents. The Biomedical Scientist **2016**; 60 (4): 209-11 summarizing conference Bacteriophage 2016, 19-21 January 2016, London

A detailed list of all my scientific, teaching and organizational achievements can be found in the Appendix 4.

**(Applicant's signature)**