SUMMARY OF PROFESSIONAL ACCOMPLISHMENTS

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1. **Name**: Agnieszka Żylicz-Stachula

2. **Diplomas and degrees with the name, place and year of their acquisition and the title of the doctoral dissertation:**

2000 Master degree in biotechnology; Intercollegiate Faculty of Biotechnology of the University of Gdansk and the Medical University of Gdansk; thesis title: "Isolation of new restriction endonuclease from thermophilic bacteria of the genus *Bacillus*"; Supervisor: Dr Stanislaw Żolnierowicz PhD

* experimental part done at Eurx Ltd. in Gdansk

2007 Doctor of biological sciences in the field of biochemistry, specialising in molecular biology; Institute of Biochemistry and Biophysics Polish Academy of Science in Warsaw; dissertation title: "Thermophilic restriction endonuclease TspGWI - a new specificity on the border of three classes of bacterial restriction-modification systems"; Supervisor: Professor Janusz Bujnicki PhD of the International Institute of Molecular and Cell Biology in Warsaw

* experimental part done at Eurx Ltd. in Gdansk

3. **Information on previous employment in scientific units:**

1999-2006 EURx Ltd - Molecular biotechnology company (a joint venture with Molecular Biology Resources Inc., Milwaukee, WI, USA), Gdansk; position: Researcher

2007-2008 Department of Theoretical Physical Chemistry, Department of Physical Chemistry, Faculty of Chemistry, University of Gdansk; position: Assistant

2008-2012 Department of Theoretical Physical Chemistry, Department of Physical Chemistry, Faculty of Chemistry, University of Gdansk; position: Assistant Professor

Since 2012 Laboratory of Genetic Engineering, Department of Molecular Biotechnology, Faculty of Chemistry, University of Gdansk; position: Assistant Professor

4. **Scientific achievement and the points received for it according to the article 16 paragraph 2 of the Act of 14 March 2003. on Academic Degrees and Titles and on Degrees and Title in Art (Journal of Laws No. 65, item 595, as amended.)**

Scientific achievement and the points received for it according to the article 16 paragraph 2 of the Act of 14 March 2003. on Academic Degrees and Titles and on Degrees and Titles in Art is a series of 7 topically related publications on restriction-modification systems derived from thermophilic bacteria belonging to the genus *Thermus*. The cycle contains 7 original works. These works were published between: 2011-2015. They cover both basic research and their application in new genetic engineering technologies and metagenomics. These publications are the result of interdisciplinary collaboration with research centres and biotechnology companies both in Poland and abroad.

The total value of the IF factor of the works constituting the achievement is 19.763. The total number of points awarded by **MNiSW** (Ministry of Science and Higher Education Republic of Poland) for the works constituting the achievement is 200.

a) The title of the scientific achievement:

Endonuclease-methyltransferases of enzyme family derived from the genus *Thermus* – comparative analysis

b) The publications included in the scientific achievement:

4.1 **Zylicz-Stachula, A***, Zebrowska J., Czajkowska, E., Wrese, W., Sulecka, E., Skowron, P.M. Engineering of TaqII bifunctional endonuclease DNA recognition fidelity: the effect of a single amino acid substitution within the methyltransferase catalytic site. *Mol Biol Rep* (2016); DOI: 10.1007/s11033-016-3949-3 (**$IF_{2014/2015}$ 2,024; $MNiSW_{2015}$ = 20**). Participation in the work: 60%.

4.2 **Zylicz-Stachula, A.**, Zolnierkiewicz, O., Sliwinska, K., Jezewska-Frackowiak, J., Skowron, P.M.* Modified 'one amino acid-one codon' engineering of high GC content TaqII-coding gene from thermophilic *Thermus aquaticus* results in radical expression increase. *Microb Cell Fact* (2014), 13:7. (**IF_{2014} = 4,221; $MNiSW_{2015}$ = 40).** Participation in the work: 50%.

4.3 **Zylicz-Stachula, A.***, Jezewska-Frackowiak, J., Skowron, P.M.* Cofactor analogue-induced chemical reactivation of endonuclease activity in a DNA cleavage/methylation deficient TspGWI N473A variant in the NPPY motif. *Mol Biol Rep* (2014), 41(4):2313-2323. (**IF_{2014} = 2,024; $MNiSW_{2015}$ = 20**). Participation in the work: 65%.

4.4 **Zylicz-Stachula, A.**, Zolnierkiewicz, O., Jasiecki, J., Skowron, P.M. * New genomic tool: ultra-frequently cleaving TaqII/sinefungin endonuclease with a combined 2.9 bp recognition site, applied to the construction of horse DNA libraries. *BMC Genomics* (2013), 14: 370. (**IF_{2013} 4,041; $MNiSW_{2015}$ = 40**). Participation in the work: 55%.

4.5 **Zylicz-Stachula, A.**, Zolnierkiewicz, O., Lubys, A., Ramanauskaite, D., Mitkaite, G., Bujnicki, J.M., Skowron, P.M.* Related bifunctional restriction endonuclease-methyltransferase triplets: TspDTI, Tth111II/TthHB27I and TsoI with distinct specificities. *BMC Mol Biol* (2012), 13:13. (**IF_{2012} = 2,796; $MNiSW_{2015}$ = 30**). Participation in the work: 45%.

4.6 **Zylicz-Stachula, A.**, Zolnierkiewicz, O., Sliwinska, K., Jezewska-Frackowiak, J., Skowron, P.M.* Bifunctional TaqII restriction endonuclease: redefining the prototype DNA recognition site and establishing the Fidelity Index for partial cleaving. *BMC Biochemistry* (2011), 12:62. (**IF_{2012} = 1,776; $MNiSW_{2014}$ = 20**). Participation in the work: 45%.

4.7 **Zylicz-Stachula, A.**, Zolnierkiewicz, O., Jezewska-Frackowiak, J., Skowron, P.M.* Chemically-induced alterations in TspGWI restriction specificity: a novel TspGWI/sinefungin endonuclease with theoretical 3-bp cleavage frequency. *Biotechniques* (2011), 50(6), 397-406. (**IF_{2011} = 2,669; $MNiSW_{2015}$ = 30**). Participation in the work: 60%.

* - by correspondence

Statements by co-authors of the publications determining the individual contribution of each author in the creation of each publication is provided in **Appendix 5**. The statements of the person

submitting the postdoctoral thesis relating to the work performed and the percentage of contribution in them can be found in **Appendix 3**.

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

Restriction-modification systems (RM) are functional, prokaryotic equivalent of immune system of eukaryotes, acting as a protection against infection by bacteriophage DNA. The discovery of restriction endonucleases (ENases) revolutionized molecular biology, influencing the development of many other areas of science and industry. Extensive research on RM systems began over 50 years ago and is still ongoing, which demonstrates the importance of this group of proteins [Loenen et al., 2014]. At the same time, these enzymes are an excellent model to study structure-function of protein – DNA interaction and test mechanisms for a specific recognition of DNA sequences. In addition, the tracking of the mechanisms and the evolution of RM systems contributes to the understanding of complex biology and mutual dependencies, operating in the exciting world of microbes, especially those that inhabit extreme environments.

In the past five years there has been rapid development of next-generation sequencing techniques (called NGS). During this period the number of deposited in the GenBank genomic DNA sequences of bacteria and archaebacteria increased more than 12.5 times. At the end of 2014, there were more than 5000 complete genomes of bacteria and archaebacterial in GenBank [Roberts et al., 2015]. Therefore, the interest in research on new RM systems, especially those belonging to the Type I and III was also increased.

Currently there are known 561 prototype specificities of Type II ENases [REBASE 20.02.16: http://rebase.neb.com]. Most of them, however, come from mesophilic bacteria. Among the commercially available Type II REases relatively few show activity at temperatures above 45°C [Gupta and Sharma, 2014; REBASE: http://rebase.neb.com]. Resistance to denaturation/thermal inactivation may be advantageous both from the point of view of stability of the enzyme during storage or enzymatic reaction as well as the use of this type of advanced molecular tools in procedures requiring higher temperatures e.g. in methods based on PCR-technology: PEAR (Polymerase - Endonuclease Amplification Reaction) [Wang et al., 2010], Thermostable Restriction Enzyme PCR Screening [Huang et al., 2006] or RTD-PCR (called Restriction Endonuclease-mediated Real-time Digestion-PCR [Zhao et al., 2013].

A rich source of thermostable ENases is bacteria belonging to the genus *Thermus*. Among them a special attention deserves, discovered by our team in 2003, the family of homologous enzymes that have ENase and methyltransferase (MTase) domain in a single polypeptide chain (Type IIC).

These enzymes are characterized by relatively rare among ENases (recognizing different DNA sequences) the similarity of the amino acid sequence (aa). For this reason, they are an excellent model to study the complex relationship between recognition sequences, protein structure and its thermal stability and evolutionary studies. The other criteria defining the above-mentioned family include molecular weight (approx. 120 kDa), the similarity of the recognition sequence and the cleavage site of DNA, the organisation of the domain, the quaternary structure (monomeric proteins in solution, may temporarily dimerize after recognizing a specific DNA sequence), the stimulation of ENase activity by S-adenosyl-L-methionine (SAM) or its analogues [Skowron et al., 2003].

A. Theoretical and experimental analysis of thermostable enzymes from the *Thermus* sp. family of proteins (Zylicz-Stachula and others, 2012)

The first gene belonging to the *Thermus* family, that I cloned, was a *tspGWIRM* gene. The results of the RM.TspGWI were presented in my doctoral dissertation and in publication Zylicz-Stachula et al., 2009, which is not a part of this scientific achievement [Zylicz-Stachula et al., 2009 erratum 2014]. In the above publication a bioinformatic analysis of RM.TspGWI aa sequence was presented, a significant similarity between aa sequences of proteins RM.TaqII and RM.TspGWI was found, a model of the protein structure was proposed, aa residues forming the catalytic centres of ENase and MTase were selected and predictions regarding the functions of key aa residues were experimentally verified. Homologous proteins RM.TspGWI and RM.TaqII consist of tandemly arranged domains, resembling the layout of structure-function subunits of Type I RM systems. Contrary to Type I enzymes, the enzymes RM.TaqII and RM.TspGWI do not have the domain of ATP-dependent translocase. Instead, they contain a centrally located helical domain and evolutionarily conserved catalytic MTase domain (like subunit HsdM of Type I RM systems). Also a common feature of these enzymes is a functional domain of nuclease (containing the catalytic motif PD-DXK), located at the N-terminus of the polypeptide. This domain is an equivalent of subunits HsdR of Type I RM systems. The results presented in the publication justified the creation of a proposed earlier by our team family of homologous proteins *Thermus* sp. [Skowron et al., 2003].

In the series of publications included in the presented scientific achievement I have isolated another prototype enzyme RM.TspDTI from the bacterium *Thermus* sp. DT and sequenced the N-terminal end of the polypeptide and proteolytic fragments of the protein. Later, on the basis of the obtained amino acid sequences I designed a primer sets. Using techniques: PCR and promiscuous PCR I amplified a segment of DNA encoding the complete gene *tspDTIRM* with flanking sequences. The obtained DNA sequence I deposited in GenBank [EF095489]. Native *tspDTIRM* gene I cloned into *Escherichia coli* (*E. coli*) bacterium under the control of a heat inducible promoter P_R from a lambda bacteriophage. I optimized the expression of the recombinant gene and designed an efficient procedure for the purification of the recombinant protein. The isolated enzyme RM.TspDTI I characterised biochemically.

Then, in collaboration with Professor Janusz Bujnicki I performed a bioinformatic analysis, which showed that the protein RM.TspDTI is a fusion protein, characterized by aa sequence similarity to enzymes RM.Tth111II, RM.TthHB27I and RM.TsoI, consisting of tandemly arranged domains: the N-terminal domain of the nuclease of the roll PD-(D/E)XK, the helical domain, the MTase domain and C-terminal domain responsible for DNA binding. Based on the theoretical analysis performed I identified the probable location of the elements of the structure responsible for the interaction with DNA. Unfortunately we were unable to clearly determine the structural details of this region. However, there were areas selected that may be responsible for the specificity of the enzyme TspDTI: WTRLAK968, PQET987 and KSMGS1028. It was also found that the C-terminal domain of the protein is composed of two TRD subdomains (Target Recognition Domain) separated by structural designs built with alpha-helices, of the type called coiled-coil (Fig. 1). In order to determine the function of these two subdomains, together with my PhD student Robert Boratyński, I constructed mutants of the gene *tspDTIRM*, devoid of the coding region TRD1 or TRD2 [**Annex 4, point. K**; doctoral thesis; Robert Boratyński, 2013]. Unfortunately, the consequence of the introduced deletions was lack of expression of mutant genes in *E. coli* bacteria, which made it impossible to clean the deletion variants of the protein for biochemical studies.

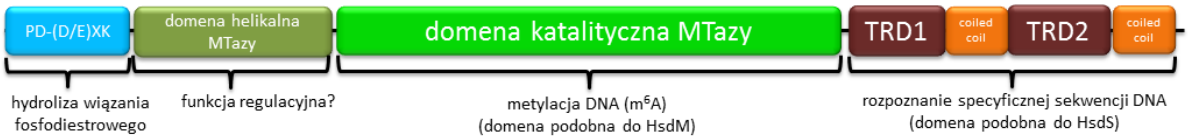

Fig. 1 Schematic representation of the domains of bifunctional enzymes belonging to the subfamily RM.TspDTI.

The above discussed organisation of domains in the RM.TspDTI protein resembles the organisation of domains in enzyme RM.TspGWI [Zylicz-Stachula et al., 2009 erratum 2014]. However, despite the substantial biochemical and recognised sequence similarity, these enzymes have limited similarity of aa sequence. Therefore, I divided the family of enzymes *Thermus* sp. into two subfamilies: a subfamily of RM.TspGWI homologous proteins and a subfamily of RM.TspDTI homologous proteins [Zylicz-Stachula et al., 2012] (Table 1). Furthermore, browsing through the available databases of restriction enzymes I found some new ENases/MTases, exhibiting significant aa sequence similarity to the thermostable enzymes from *Thermus* sp. family (Table 1) [Zylicz-Stachula et al., 2012; Skowron et al., 2013].

ENase	Source	Recognised sequence	Cleavage	Optimum reaction temperature	Molecular Weight (Da)
Subfamily RM.TspGWI					
RM.TspGWI	*Thermus sp.*	ACGGA	$N_{11/9}$	65-70ºC	120 201
RM.TaqII	*Thermus aquaticus*	GACCGA	$N_{11/9}$	65-70ºC	125 674
RM.TaqIII	*Thermus aquaticus*	CACCCA	$N_{11/9}$	65-70ºC	~ 120 000
RM.RpaI	*Rhodopseudomonas palustris*	GTYGGAG	$N_{11/9}$	37ºC	119 291
Subfamily RM.TspDTI					
RM.TspDTI	*Thermus aquaticus*	ATGAA	$N_{11/9}$	65-70ºC	126 885
RM.Tth111II/TthHB27I	*Thermus thermophilus*	CAARCA	$N_{11/9}$	65-70ºC	125 955
RM.TsoI	*Thermus scotoductus*	TARCCA	$N_{11/9}$	55ºC	126 474
RM.CchII	*Chlorobium chlorochromatii*	GGARGA	$N_{11/9}$	37ºC	121 699
Heat labile enzymes potentially homologous to the enzymes from *Thermus* family, not qualified to a specific subfamily because of the lack of available aa sequence					
EciI	*E. coli*	GGCGGA	$N_{11/9}$	37ºC	~ 120 000
RleAI	*Rhizobium leguminosarum*	CCCACA	$N_{12/9}$	26ºC	-

Table 1 The enzymes belonging to the *Thermus* family. The gray marked enzymes come from mesophilic bacteria.

Most of these newly selected enzymes (except for RM.TsoI) come from mesophilic organisms, which is another proof of the existence of effective mechanisms of horizontal gene transfer between microorganisms inhabiting isolated, extreme environments and microorganisms living at lower temperatures in more complex ecosystems. Thermophilic microorganisms strategy relies on effective adaptation to functioning in extreme conditions, which significantly minimize competition. Such an adaptation also occurs due to the efficient acquisition of "foreign" genes important for thermophilic bacteria from the point of view of their metabolism, protection against bacteriophages or to gain an advantage over other microorganisms with which they must compete. Bacteria of the genus *Thermus* are characterized by the so-called natural competence [*Thermus* species (Biotechnology Handbooks) Sharp.R and Williams.R, eds., 1995], which means they are able to receive and hold in the genome large fragments of exogenous DNA. This natural

competence depends on the products of at least 16 different genes [Friedrich et al., 2001, 2002], for both genomic and plasmid DNA. In the genomes of these bacteria there were also found many functional interspecific and intraspecific paralogs. It was also shown that bacteria of the genus *Thermus* have so called magaplasmids e.g. in bacteria *Thermus aquaticus* (*T. aquaticus*) 4-5 different megaplasmids were found [*Thermus* species., Sharp R. and Williams R., eds., 1995]. One of the described in the literature examples of such plasmids are megaplasmids isolated from *Thermus thermophilus* HB8 and HB27, which include, among others, genes responsible for adapting to life in extreme temperatures, encoding different schemes for DNA repair [Bruggeman and Chen, 2006]. An interesting, for me, aspect of the bacteria biology of the genus *Thermus* is the coexistence of the natural competence mechanisms along with numerous functional restriction-modification systems, which protect these bacteria against exogenous DNA. Another interesting puzzle to solve is how these two opposing mechanisms may act antagonistically in a single bacterial cell.

B. The cloning of native and synthetic gene *taqIIRM* - developing a strategy to increase the level of expression of genes encoding thermostable ENases in the *E. coli* bacteria (Zylicz-Stachula et al., 2014)

Because of its origin, the number of base pairs, and the characteristic for bacteria of the genus *Thermus* codon usage method, the native genes encoding bifunctional ENases/MTases of the *Thermus* sp. enzyme family are not highly expressed in *E. coli*. Moreover, these genes are relatively difficult to clone because they encode enzymes with ENase activity, potentially "toxic" for bacterial host cell. The accompanying MTase activity is not sufficient to protect the DNA of the genomic host at the time of installation of the RM system in the cell (unpublished data). It is also unknown how the level of both activities located in a single polypeptide is controlled. To get around the problem of low expression and to obtain the right amount of protein for further study, a modified codon optimization strategy (one amino acid-one codon) was used. For this purpose a gene with a length of 3315 bps was designed and synthesized. This gene encoded the enzyme RM.TaqII. The synthetic gene was characterized by a significantly reduced % (percentage) of GC pairs and a limited number of mRNA secondary structures. From 1105 codons as many as 718 (65%) were changed to increase expression in *E. coli*. A similar approach has not been previously described in the literature in the context of cloning ENase encoding genes. The applied strategy of "one amino acid-one codon" was chosen deliberately because of our concerns that an excessive expression of the recombinant gene, and the large overproduction of the protein it entails, could lead to a rapid lysis of recombinant *E. coli*. For this reason, I decided to use the strategy described in the literature as less effective compared to the strategy of codon randomization, involving the random selection of codons from a pot of the most common in the genome, or the most commonly occurring in the genes with high expression [Menzela, 2011]. Native and synthetic variant of the *taqIIRM* gene were cloned into *E. coli* bacteria by myself in collaboration with my PhD student Olga M. Żołnierkiewicz (**Annex 4, point K**), based on the inducible expression system that uses temperature promoter P_R from lambda bacteriophage previously tested for homologous TspGWI protein [Zylicz-Stachula et al., 2009]. The bacterial cultures growth, before the induction was carried out at 28°C, in order to minimize the possible activity of TaqII ENase resulting from the potential "leaks" of the used expression system. After the induction, the cultures were grown at 42°C to allow proper folding of the protein. As a result of the strategy used I obtained a significant increase in the expression of the optimized gene *taqIIRM*, compared to the intact gene, yielding over 10 times more protein RM.TaqII per 1g of bacterial mass. In addition, I presented, in the above discussed paper, an efficient procedure for the purification of the recombinant protein, I marked activity of ENase and MTase, analysed the biochemical properties of the recombinant protein, including establishing the optimum reaction temperature of recombinant TaqII ENase.

C. Verification of the recognition sequence of the enzyme RM.TaqII (Zylicz-Stachula et al., 2011)

One of the most puzzling prototypical enzymes belonging to the *Thermus* family is TaqII [Barker et al., 1984; Zylicz-Stachula et al., 2011, 2014]. This bifunctional enzyme (having a REase and MTase domain in a single polypeptide chain - Type IIC) is a protein homologous to RM.TspGWI. Aa sequence similarity between these two enzymes is one of the highest reported so far in the literature of sequence similarities between ENases, recognizing different DNA sequences [Zylicz-Stachula et al, 2009; Erratum 2014].

According to the Barker et al. publication, native TaqII ENase is capable of specifically recognizing two variants of 6-nt (nucleotides) DNA sequence: 5'-GACCGA-3 'and 5'-CACCCA-3'.

In the publication Zylicz-Stachula et al., 2011, which is a part of this scientific achievement, I showed that contrary to the native enzyme the recombinant variant only recognizes the sequence 5'-GACCGA-3 '. Despite the observed difference in the recognition sequence, I found no change in the place of DNA cut. In both cases, the hydrolysis of the phosphodiester bond always happened in a strictly defined distance of 11 and 9 nt from the recognition sequence to form a two-nucleotide (nt) 3'-protruding sticky ends. In the case of the recombinant enzyme RM.TspGWI, despite a significant similarity to aa sequence of RM.TaqII, I did not observe a difference in the specificity of the recognition sequence between the native and recombinant protein variant [Zylicz-Stachula, 2002, 2009]. What is more, a similar phenomenon has not yet been described in the literature for any of the known ENases.

In order to clarify this interesting problem I developed several hypotheses. The first assumed that in the bacteria *T. aquaticus* YT-1 there is an additional RM system, which includes a new prototype ENase which recognizes the sequence 5'-CACCCA-3 '. So far, there are two Type II RM systems of *T. aquaticus* bacteria described in the literature: TaqI - recognizing the sequence 5'-TAGC-3 ' [Sato et al., 1977] and RM.TaqII [Barker et al., 1984; Zylicz-Stachula et al., 2011, 2014]. The second hypothesis assumed that the recombinant variant of the protein TaqII may differ considerably in structure from the native variant due to incorrect folding t post-translational modifications of the protein in the mesophilic bacteria *E. coli*. Such hypothetical structural differences may result in loss of recognition of one of the two variants of the DNA sequence. The third hypothesis was based on the existence of an unknown additional subunit whose presence could condition the recognition of the 5'-CACCCA-3' sequence. To verify the above mentioned hypotheses I tried to get funding for the implementation of this project by applying for grants in the NCN (National Science Centre Poland) competitions (**Annex 4, point Q**).

Despite limited financial resources, together with Dr Joanna Makowska and Ms Joanna Żebrowska MSc, a PhD student, of whom I am a supervising tutor, we carried out preliminary studies on the structure of the recombinant protein TaqII (CD, DSC, ANS-protein complexes fluorescence research). Furthermore, in collaboration with Assoc. Prof. Mathias Bochtler I made my first attempt to crystallize the TaqII: DNA complex in the presence of SIN and SAH. I also obtained a draft version of the genome sequence of *T. aqiaticus* YT-1, which was deposited in GenBank [LHCI01000000] (**Annex 4, point Q**).

In addition, I was able to isolate in the bacterium *T. aquaticus* YT-1 the next bifunctional ENase/MTase recognizing the sequence 5'-CACCCA-3 'and cutting DNA at a distance of 11 and 9 nt from the recognition sequence, which confirmed my first research hypothesis. The isolated RM.TaqIII (Table 1) is a protein having a molecular weight of about 120 kDa, as RM.TaqII enzyme and other enzymes belonging to the *Thermus* sp enzyme family. RM.TaqIII is most probably a

protein encoded by a functional paralog of the *taqII*RM gene which is in the genome of *T. aquaticus* YT-1. According to the recent reports in the literature *Thermus* bacteria are characterized by extremely plastic, polyploid genome, which facilitates the repair of the damage sustained in high temperatures [Ohtani et al., 2010].

This means that all the key for the bacteria genes are present in the cell in several copies. In the course of the *T. aquaticus* YT-1 bacteria's evolution, in one copy of the gene *taqIIRM* in the area encoding the TRD domain probably spontaneous mutations happened which led to the change of the recognition sequence. As a result of these mutations another variant of the enzyme appeared (very similar biochemically to RM.TaqII). These findings and hypotheses I have presented at the 7th NEB Meeting 2015 in Gdansk (**Annex 4, point B,** publication in preparation). Further research on these unique RM systems I have been carrying out in cooperation with world-class specialists in the field of RM systems: Prof. Sir Richard Roberts, Dr Goeffrey Wilson, Dr Richard Morgan and Dr Alexey Fomenkov from New England Biolabs, whom I met during the above-mentioned conference.

D. Chemically induced, controlled relaxation of the recognition sequence (Zylicz-Stachula et al., 2011; Zylicz-Stachula et al., 2013)

One of the most interesting aspects of the biology of the ENases/MTases family researched by our team is unique but also varied modulation of enzymatic activity under the influence of SAM or its analogue sinefungin (SIN). In addition to functioning as an allosteric effector SAM is also a methyl group donor in methylation of DNA. Stimulation or inhibition of the activity of ENase by SAM has been described previously in the literature and is characteristic for many enzymes belonging to Type IIC/IIG. The allosteric effector function may also be taken by various SAM analogies, for example SIN or S-adenosylhomocysteine (SAH). These compounds play a role in signalling the switch between modification and restriction mode affecting the conformational change in the enzyme associated with the simultaneous change of protein interactions with the various principles of the recognition sequence. Among the tested ENases from the *Thermus* sp. enzyme family no ENases showed activity under SAH stimulation. All tested ENases were, however, strongly stimulated in the presence of SIN. A similar effect I observed in the case of SAM stimulation, which resulted in an increase in activity of the majority of tested ENases: RMTspDTI, RM.TaqII, RM.Tth111II, RM.TthHB27I and RM.TsoI, acting as a suppressant only in the case of RM.TspGWI - an enzyme homologous to RM.TaqII.

In the above presented series of works I analysed the recognition sequences and biochemical properties of two prototype ENases: TspGWI and TaqII in the presence of SAM and SIN [Zylicz-Stachula 2011, 2013]. During these studies I demonstrated that enzymes from the *Thermus* sp. family have the unique ability to relax the recognition sequence, induced by the presence of SIN in the reaction mixture [Zylicz-Stachula 2011, 2013]. In the presence of SIN RM.TspGWI and RM.TaqII recognize the degenerate variants of DNA sequences which differ by 1 nt from the canonical sequence. I found that the relaxation may affect any nt within the recognition sequence. Another factor which intensifies the degree of relaxation of the recognition sequence is DMSO. For RM.TaqII the combination SIN/DMSO results in the recognition of sequence variants which differ from the canonical sequence by two nt. The DNA cleavage site, however, does not change in neither TspGWI nor TaqII. In these works I showed that in the presence of SIN TspGWI ENase changes its specificity from 5-nt sequence to the equivalent of 3-nt DNA sequence. Whereas the specificity of TaqII ENase can be converted under the influence of the SIN/DMSO combination from 6-nt sequence to the equivalent of 2.9-nt sequence. Both ENases in the presence of SIN are converted to ultra-frequent DNA cleaving enzymes [Zylicz-Stachula 2011, 2013], corresponding in the frequency of DNA cutting to the unique CviJI / CviJI *, SetI and FaiI ENases (REBASE: http://rebase.neb.com). The chemically induced change in the sequence specificity

recognized by RM.TspGWI and RM.TaqII, discovered by us, was further used for practical purposes - the creation of new, ultra-frequent DNA cutting molecular tools designed to construct representative, randomized genomic libraries. The usefulness of the new molecular tools I demonstrated through the use of the ENase activity of TaqII/SIN/DMSO for the construction of the representative genomic DNA library of a domestic horse (*Equus caballus*) [Zylicz-Stachula et al., 2013]. Currently, genomic technologies and mass sequencing projects (including a group of genome sequencing projects of all organisms on Earth) are one of the leading branches of biology and certainly the next few decades will pass under the sign of bioinformatics cataloguing biodiversity. Thus, our study of enzymes from the *Thermus* family generate not only the knowledge of enzymology of these highly unusual ENases/MTases, but also is an important scientific contribution to broadening the base of overall biology knowledge.

It is worth mentioning that SIN is a naturally occurring antibiotic isolated from *Streptomyces incarnatus* NRRL 8089 and *Streptomyces griseolus* [Yadav et al., 2014]. This compound has a potent anti-fungal, antiviral and antiprotozoal effect [Fukuda et al., 2010]. Recently it has also been shown the inhibitory effect of the antibiotic on the formation of *Streptococcus pneumoniae* biofilm *in vitro* and the ability of these bacteria to colonize *in vivo* [Yadav et al, 2014]. An interesting aspect of the studied phenomenon of the recognition sequence relaxation of ENase under the influence of SIN could be a synergistic, anti-bacteriophage SIN result, which effectively limits infections. There are known thermophilic *Streptomyces* species that inhabit the same ecological niche as bacteria of the genus *Thermus*. However, SIN biosynthesis genes derived from thermophilic microorganisms have not been described in the literature yet. Thus, it is not known whether SIN may be a compound naturally occurring in the environment inhabited by bacteria of the genus *Thermus*.

E. The development of a new strategy for engineering bifunctional ENases/MTases towards enzyme variants with improved properties by site-specific mutagenesis in the region encoding the motif IV MTase (motif NPPY) (Zylicz - Stachula et al., 2014; Zylicz-Stachula et al., 2015)

During the experimental verification of predictions about the functions of individual amino acid residues forming the catalytic centres of RM.TspGWI and RM.TaqII I noticed that the substitutions of asparagine (respectively N473 or N472) by alanine resulted in an interesting change in the activity of the enzymes. The aforementioned asparagine is one of the aa in the evolutionarily preserved motif IV of MTase (motif NPPY). The consensus sequence of this motif is: (S/N/D)PP(Y/F/W). This asparagine may be, as in TaqI MTase [Goedecke et al., 2001], responsible for the formation of a hydrogen bond with an amino group of adenine substrate contained in a recognition sequence. Adenine is "unlooped" from the double-stranded helix of a DNA. Second, weaker hydrogen bond is formed between proline and adenine. For RM.TspGWI the substitution of asparagine by alanine resulted in a protein variant lacking the activity of ENase and MTase. However, in the presence of SIN the tested ENase activity was restored, reaching approximately 25-50% of the intact protein activity, depending on the DNA substrate [Zylicz - Stachula et al., 2014]. For RM.TaqII the analogous substitution of asparagine by alanine caused a different effect. The resulting protein variant retained limited MTase activity (about 8 times less than the intact enzyme). At the same time the ENase activity under standard reaction conditions was almost completely eliminated, regardless of the presence of SAM or SIN. A slight change of reaction conditions, however, the increase of the reaction temperature by 5°C and changing pH by 0.5 unit, led to the SIN dependent restoration of the activity of ENase at 25% of the intact protein activity. The resulting variant of TaqII had at the same time greatly increased accuracy of DNA sequence recognition [Zylicz-Stachula et al., 2015]. These results demonstrated that the asparagine at position 473/472 is essential for the modulation of the two enzymatic activities of proteins from the *Thermus* family. Based on these results I formulated a hypothesis that there is a possibility of engineering new variants of IIC/IIG/IIS enzymes of the *Thermus* family with improved properties

by substituting the asparagine in the NPPY motif. In order to verify this hypothesis, together with Ewa Sulecka (a PhD student, of whom I am a supervising tutor; **Annex 4, point K**) we carried out a saturation mutagenesis at alanine 472 codon of the protein RM.TaqII. We obtained a number of mutants, several of which we chose for optimizing expression, isolation of protein variants and further biochemical studies. While choosing we took into consideration the properties of amino acids substituted in the 472 position. As a result, we received three protein variants of RM.TaqII with several times increased activity of ENase and MTase compared to the intact variant. In contrast to the intact enzyme the variants had the ability to completely digest the DNA substrate. These results were presented at the 7th NEB Meeting 2015 in Gdansk (**Annex 4, point B**, publication in preparation).

To summarise, the presented work is an important contribution to deepening the knowledge of thermophilic bacteria of the genus *Thermus* biology and the unusual RM systems operating in these bacteria.

Up to the present, our team has already published fourteen works about enzymes from the *Thermus* family, seven out of which are part of this scientific achievement. Other works were not included either due to my lesser contribution in them or the fact that part of the published results was used by me in my doctoral thesis (**Annex 3, point II.A**).

Four new works of which I am a co-author and which concern the same topic are now being either under review or prepared.

I believe my most important scientific achievements are:

• The verification of the recognition sequence of the enzyme RM.TaqII, the solution of the problem of the observed differences in the sequences recognized by the native and recombinant variant of RM.TaqII, and the discovery and isolation of RM.TaqIII.

• The establishing of the aa sequence of RM.TspDTI. A bioinformatic analysis of aa sequence, and the separation of two subfamilies within the *Thermus* family. Extention of the *Thermus* family by enzymes from mesophilic bacteria.

• The development of a method allowing a significant increase in expression of genes encoding thermostable ENases/MTases in *E. coli*.

• The discovery of chemically-induced, controlled relaxation of the recognition sequence of ENases from the *Thermus* enzyme family and the creation of a new molecular tool for the construction of representative genomic libraries.

• The development of a new strategy for engineering bifunctional ENases/MTases resulting in the enzyme variants with improved properties through site-specific mutagenesis in the region encoding the motif IV MTase.

5. The review of other scientific and research achievements:

The scientific output (**detailed in Annex 3**)

The total number of full-text publications (with the exclusion of the cycle of works for the postdoctoral thesis): 12 (including 3 works of which I was the first author), the sum of points awarded by IF = 41,284, $MNiSW_{2015}$ = 375,
including:

- 2 pre-doctoral publications (including 1 work of which I was the first author) IF = 13.625, MNiSW = 80;
- 9 post-doctoral publications (including 2 works of which I was the first author; IF = 27.659, MNiSW = 295)

Postdoctoral publication cycle: 7 publications (including being the first author of seven works, and author by correspondence of 2 works) with IF = 19.763, MNiSW = 200.

The total number of full-text publications (including the works from the postdoctoral publication cycle): 19 (including being the first author of 10 works, the author by correspondence of 2 works), IF = 60,72, MNiSW = 575.

The total number of congress abstracts 40 (0 before doctoral degree, 40 after obtaining doctorate): 27 at international conferences and 13 at national conferences.
The works have been cited 87 times, and excluding self-citation 25 times. Hirsch index is 6 (according to the Web of Science from 22.02.2016).

Research projects after obtaining the doctoral degree as a manager or contractor:

UG-BW 8000-5-0143-8 (2008): "The influence of S-adenosyl-L-methionin, Sinefungin and S-adenozyl-L-homocysteine on restriction endonuclease TspGWI activity - experimental studies" (project manager).

UG-BW 8000-5-0265 (2009): "The mechanisms regulating the activity of a restriction endonuclease TspGWI - experimental studies" (project manager).

KBN / N 204 023 135 (2008-2011). "Photoreactivity and radioactivity research of DNA molecules modified by halogen derivatives of nucleic bases. Experimental and quantum chemical studies"(contractor).

MNiSW N 204 156040. (2011-2014). "Photo- and radiosensitizing of DNA using the bromo derivatives of nucleic bases" (contractor).

NCN, OPUS, UMO-2012/05/B/ST5/00368 (2013-2016). "The impact of the nucleotide sequence on the performance of radio- and photodamage of double-stranded DNA fragments labelled with halogen derivatives of nucleic bases" (contractor - 2013).

Research and development projects with practical purposes carried out as a contractor after obtaining the doctoral degree:

NCBiR development project in the field of biotechnology NR12-0070-06/2009 (2009-2013).
"The development of technology for industrial production of recombinant cholinesterases based on molecular cloning of genes encoding enzymes" (main contractor).

EU3.1 project. Initiating the activities of Operational Programme Innovative Economy (2007-2013) UDA-POIG.03.01.00-00-010/10-00: "The development of technology for the production of third-generation hydrogel dressing on the basis of alginates and biologically active substances introduced into the dressing.", funded by Nickel Technology Park Poznan for the special purpose vehicle MedVentures Ltd. in collaboration with UG (main contractor).

Investment Grant (industrial) of Operational Programme Innovative Economy 1.4, EU/NCBiR: POIG.01.04.00-22-140/12: "The new technology of producing preventive and therapeutic

vaccines for multiple stimulation of the human immune system " for the special purpose biotechnology vehicle BioVentures Institute Ltd. in collaboration with UG (main contractor).

NCBiR STRATEGMED Regennova Grant (2014-2017) "New technologies for pharmacological stimulation of regeneration" carried out by a consortium University of Gdansk, Medical University of Gdansk, Gdansk University of Technology, the Nencki Institute of Experimental Biology PAN, MedVentures Ltd. and Pro-Science Poland Ltd. (contractor).

The course of scientific work before the doctoral degree

I began studying in 1995 at Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdansk (MWB UG-GUMed). While still a student I conducted research activities as part of several individual student internships, carried out in national and international centres. My own scientific work began during the fourth year of studies as part of a scientific internship at a biotechnological company Eurx Ltd. In 1999, after completing a scientific holiday internship, I received an offer from Prof. Piotr Skowron of a position as a researcher in the aforementioned company and the possibility to carry out the experimental part of my master's thesis as part of my project to look for new thermostable restriction endonucleases with the potential for industrial use. The results of the thesis, entitled "The isolation of new restriction endonucleases: BspGWI and BspTUI from the thermophilic bacteria of the genus *Bacillus*," I presented at the 8th International Students Conference in 2000 in Gdansk.

In 2000 I graduated with a first class Master's degree and started further work in Eurx Ltd. where I stayed until November 2006.

While working at Eurx I was the main contractor of the multiannual program seeking new thermophilic restriction endonucleases. The result of this project was my discovery of two restriction endonucleases with new specificities: TspGWI and TspDTI and many thermophilic isoschizomers of known restriction enzymes. This discovery was at the time unique on a global scale; it accounted for 50% of new specificities discovered worldwide in 2002. As a result of these scientific achievements I received an informal status of an independent researcher at Eurx Ltd.

Between: 2001-2006 I conducted independently many research projects on: *(i)* cloning and expression of various prokaryotic and eukaryotic genes, *(ii)* protein engineering, as well as isolation of native and recombinant bacterial/viral proteins, including a number of restriction endonucleases that are currently Eurx Molecular Biotechnology Ltd.'s commercial offer (http://www.eurx.com.pl). These projects were discussed in detail in **Appendix 3; point II.I.**

Further studies on the prototype enzymes RM.TspGWI and RM.TspDTI found during my fifth year of college resulted in the discovery of a new family of restriction nucleases type *Thermus*, two original publications in the Nucleic Acids Research journal, and later on my doctoral dissertation. I would like to mention that due to the corporate policy of the biotechnology company where I was employed the above-mentioned works were unfortunately the only works published by our team between: 2000-2006. Such policies, that avoid publishing of the results due to the absence of patent protection, lead, unfortunately, to the reduction of possibilities of scientific development of young researchers employed in the industry. The unpleasant consequences of this disadvantageous policy I feel to this day, especially during subsequent attempts to obtain funding for my own research in NCN and FNP competitions.

With a desire to further pursue my passion for science in 2006, I made a very difficult for me, life-changing decision, to abandon the lucrative position in the biotech company. I established cooperation with Prof. Janusz Bujnicki of the International Institute of Molecular and Cell Biology

in Warsaw. From November 2006 to November 2007, with full support and approval of Prof. Piotr Skowron, I was preparing a doctoral thesis, in which I partly used the scientific results obtained while working for Eurx.

The result of the work carried out as part of my doctorate are the following publications (one of them was published after I defended doctoral dissertation):

- **Zylicz-Stachula A**., Bujnicki, J.M., Skowron, P.M. Cloning and analysis of bifunctional DNA methyltransferase/nuclease TspGWI, the prototype of a *Thermus* sp. family. *BMC Mol. Biol.,* (2009), 10, 52. Erratum in *BMC Mol Biol* 2014;15:16. (**IF_{2009} = 2,848; $MNiSW_{2015}$ = 30**).
- Skowron, P.M., Majewski, J., **Zylicz-Stachula, A.**, Rutkowska, S.R, Jaworowska, I., Harasimowicz-Słowińska, R. A new *Thermus* sp. class-IIS enzymes subfamily: isolation of a "twin" restriction endonuclease TspDTI, with a novel specificity 5'-ATGAA(N11/9)-3' related to TspGWI, TaqII and Tth111I. *Nucleic Acids Res* (2003), 31, e74. (**IF_{2003} = 6,575; $MNiSW_{2015}$ = 40**).
- **Zylicz-Stachula, A.**, Harasimowicz-Słowińska, R., Sobolewski, I., Skowron, P.M. TspGWI, a thermophilic class-IIS restriction endonuclease from *Thermus* sp. recognizes novel asymmetric sequence 5'-ACGGA(N11/9)-3'. *Nucleic Acids Res* (2002), 30, e33 **(IF_{2002} 7,051; $MNiSW_{2015}$ = 40)**

The Dissertation entitled "Thermophilic restriction endonuclease TspGWI - a new specificity on the border of three classes of bacterial restrictive-modification systems" I prepared under the supervision of Prof. Janusz Bujnicki, and defended at the Institute of Biochemistry and Biophysics Polish Academy of Science (PAN) in Warsaw in November 2007, receiving the degree of Doctor of Biological Sciences in the field of biochemistry. The reviewers of my work were Prof. Andrzej Piekarowicz and Prof. Zofia Szweykowska-Kulińska. My doctoral thesis concerned the isolation of the native enzyme TspGWI, sequencing, bioinformatic analysis, cloning and gene expression of *tspGWIRM* gene in *E. coli* bacteria and comprehensive biochemical analysis of native and recombinant variant of the studied protein.

The course of scientific work after receiving the doctoral degree

After obtaining the doctoral degree I started working in the Division of Theoretical Physical Chemistry (now Laboratory of Biological Sensitizers) in the Department of Physical Chemistry at the Faculty of Chemistry UG, led by Prof. Janusz Rak, wherein I participated in interdisciplinary studies on radio- and photosensitizers of DNA, in order to selectively sensitize tumor cells to high-energy and UV radiation. During my work in the Division of Theoretical Physical Chemistry I participated in the creation and organization of a new scientific laboratory, training my co-workers (students and PhD students of the Faculty of Chemistry UG) in contemporary techniques of molecular biology and genetic engineering, which we introduced to the study of radio- and photosensitizers of DNA. Moreover, I had a significant role in preparing 3 research grants (listed in **Annex 3, point II.I**), which enabled Prof. Janusz Rak's team to purchase basic laboratory equipment and start the planned interdisciplinary experimentation.

The results of our joint research were published:

- **Zylicz-Stachula, A**., Polska, K., Skowron, P.M., Rak, J. Artificial Plasmid Labeled with 5-Bromo-2'-deoxyuridine: A Universal Molecular System for Strand Break Detection. *ChemBioChem* (2014), 15(10):1409-1412. (**IF_{2014} = 3,088; $MNiSW_{2015}$ = 30**)

- Zdrowowicz, M., Michalska, B., **Zylicz-Stachula, A**, Rak, J. Photoinduced Single Strand Breaks and Intrastrand Cross-Links in an Oligonucleotide Labeled with 5-Bromouracil. *J Phys Chem B.* (2014), 118(19):5009-5016. (**IF_{2014} = 3,302; $MNiSW_{2015}$ = 30**)

- Sobolewski, I., Polska, K., **Zylicz-Stachula, A.**, Jezewska-Frackowiak, J. Rak J., Skowron, P. Enzymatic synthesis of long double-stranded DNA labeled with haloderivatives of nucleobases in a precisely pre-determined sequence. *BMC Biochemistry* (2011), 12:47. (**IF_{2012} = 1,988; $MNiSW_{2015}$ = 20**).

- Michalska, B., Sobolewski, I., Polska, K., Zielonka, J., **Zylicz-Stachula, A.**, Skowron, P., Rak, J. PCR synthesis of double stranded DNA labeled with 5-bromouridine. A step towards finding a bromonucleoside for clinical trials. *J Pharm Biomed Anal* (2011), 56, 671-677. (**IF_{2011} = 2,967; $MNiSW_{2014}$ = 35**)

- Polska, K., Zielonka, J., Chomicz, L., Czerwicka, M., Stepnowski, P., Guzow, K., Wiczk, W., Smuzynska, M., Kasprzykowski, F., **Zylicz-Stachula, A.**, Skowron, P.M., Rak, J. Unexpected photoproduct generated via the acetone-sensitized photolysis of 5-Bromo-2'-deoxyuridine in a water/isopropanol solution: experimental and computational studies. *J. Phys Chem B* (2010), 114, 16902-16907. (**IF_{2010} = 3,603; $MNiSW_{2014}$ = 30**).

While working in Prof. Janusz Rak's team, I remained, closely linked to the subject I had been interested in since my time at university that is the thermostable restriction endonucleases. With the consent of Prof. Janusz Rak about 50% of my working time I devoted to the study of enzymes belonging to the discovered by us *Thermus* family, planning to use the published works to prepare my own habilitation thesis. I continued my research in collaboration with Prof. Janusz Bujnicki and Prof. Piotr Skowron, who was my superior during the period of work in Eurx company and who since 2006 has been employed at the Department of Chemistry UG. In 2010, while searching online databases I found two more enzymes from mesophilic bacteria, RM.RpaI and RM.CchII as well as one enzyme RM.TsoI isolated from thermophilic *Thermus scotoductus*, which, due to their significant amino acid sequence similarity I classed as belonging to the discovered by us *Thermus* sp enzyme family. As a result of this discovery our team established scientific cooperation with Prof. Arvydas Lubys (Director of Research and Development at Thermo Fisher Scientific Baltics UAB, Department of Botany and Genetics, Vilnius University, Lithuania), concerning RM.TsoI. This cooperation has so far resulted in three publications:

- Zylicz-Stachula, A., Zolnierkiewicz, O., Lubys, A., Ramanauskaite, D., Mitkaite, G., Bujnicki, J.M., Skowron, PM. Related bifunctional restriction endonuclease-methyltransferase triplets: TspDTI, Tth111II/TthHB27I and TsoI with distinct specificities. *BMC Mol Biol* (2012), 13:13. **(IF_{2012} = 2,796; $MNiSW_{2014}$ = 30)**

- Skowron, PM., Vitkute, J., Ramanauskaite, D., Mitkaite, G., Jezewska-Frackowiak, J., Zebrowska, J., Zylicz-Stachula, A., Lubys, A. Three-stage biochemical selection: cloning of prototype class IIS/IIC/IIG restriction endonuclease-methyltransferase TsoI from the thermophile *Thermus scotoductus*. *BMC Mol Biol.* (2013), 14:17. (**IF_{2013} = 2,057; $MNiSW_{2014}$ = 30**)

- Jezewska-Frackowiak, J., Lubys, A., Vitkute, J., Zakareviciene, l., Zebrowska, J., Krefft D, Skowron, M., Zylicz-Stachula, A., Skowron, P.M.: A new prototype IIS/IIC/IIG endonuclease-methyltransferase TsoI from the thermophile *Thermus scotoductus*, recognizing 5'-TARCCA(N11/9)-3' sequences. *J. Biotechnol.* (2015), 194:19-26. (**IF_{2015} = 2,884; $MNiSW_{2014}$ = 30**)

In addition, since 2007 together with Prof. Piotr Skowron and Dr of Engineering Joanna Jeżewska-Frąckowiak we had been creating a new Department of Molecular Biotechnology, organizing from scratch a modern and dynamically operating laboratory of molecular biology, applying for appliance and research-and-development grants, developing cooperation with foreign and domestic research centres, as well as training chosen students and doctoral students of the Faculty of Chemistry, interested in interdisciplinary research on the borders of chemistry and molecular biology, who soon became valuable members of our team.

In September 2012 I received the offer of official transfer to the co-created by me Department of Molecular Biotechnology, led by Prof. Piotr Skowron, where I work to this day. Due to my specified research interests, but above all due to the time constraints associated with the large number of research projects carried out, I decided to end my previous scientific cooperation with Prof. Janusz Rak. My decision was also influenced to a large extent by the fact that the team of Prof. Janusz Rak already included well-trained, partly by me, staff, who could successfully step in for me and continue research work.

I am continually trying to keep a close relationship with the biotechnology industry through internships and collaboration with biotech companies (REVONGEN, New England Biolabs, Thermo Fisher Scientific Baltics UAB, MedVentures, BioVentures, Innovabion, ProScience), as well as participation in numerous application projects (**Annex 3, point II.I**).

6. Research plans:

At present, I am taking an active part in two major R & D projects concerning modern methods of construction of a new generation of recombinant vaccines and regenerative medicine (**Annex 3, point II.I**). However, the immediate subject of my research, carried out since 2000, remain the unique ENases-MTases of the *Thermus* enzyme family. This project, in contrast to other carried out by me application studies is purely cognitive. So far, it has resulted in 14 publications, of which I am a co-author, in journals of the Journal Citation Reports database. Subsequent publications are currently in preparation. This project has still large publishing potential and arouses widespread interest of the recognized experts in the field of ENases, what I had the opportunity to observe while presenting the results of my research at taking place once every four years international conference: 7th NEB Meeting on DNA Restriction and Modification in Gdansk in August 2015 (**Annex 4, point B**), where I was invited to give a presentation. Despite the existing difficulties in obtaining in Poland funds for the research of these topics (so far I have submitted four grant applications in competitions NCN and one in FNP, which are listed in **Annex 4, point F**) I hope I will get the opportunity to continue the research as part of my own scientific project. In 2014, I established cooperation with Prof. Matthias Bochtler of the International Institute of Molecular and Cell Biology in Warsaw, within this collaboration I made the first attempts to crystallize the recombinant protein RM.TaqII in complex with DNA, and SIN or SAH, which are the analogues of SAM – the cofactor of TaqII MTase. The enzymes from *Thermus* group are very difficult proteins for crystallographic studies due to their molecular weight (about 120 kDa) and the presence of the mobile domain of ENase. So far no one has managed to get the structure of any representative of thermostable enzyme of this type. Currently we are preparing a deletion variant of TaqII protein which has an MTase activity, but is devoid of ENase domain, to increase the chance of securing a sufficient quality of stable crystals of a protein-DNA complex. We were able to construct such variant already. At the moment we are still working on the optimization of the recombinant gene expression and purification of the adequate amount of the protein for further crystallographic studies.

In addition, I intend to continue to pursue the research of restriction-modification systems found in bacteria *T. aquaticus* YT-1: RM.TaqII and discovered recently by me RM.TaqIII, in the

context of the existing in these bacteria unique evolutionary mechanisms that lead to the creation of new protein variants with altered specificity of DNA recognition sequence. I'm interested in the interrelationships and the manner of regulation of gene expression forming the above-mentioned systems of the RM, as well as verification of subsequent, formulated by me, research hypothesis concerning the existence of unknown mechanisms functioning in bacteria of the genus *Thermus*, working at the transcriptional level of the tested genes or at the level of post-translational processing of the tested proteins leading to changes in the recognition sequence specificity and consequently to the increase in the number of functional ENases in bacterial cells. These projects I intend to carry out in cooperation with New England Biolabs (USA).

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