Institute of Biotechnology
Centre of Excellence

Biennial Report 2006 - 2007

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Founded in 1975 as the All Union Research Institute of Applied Enzymology, currently, IBT is mainly involved in research and training in the fields of biotechnology and molecular biology, including research and development of recombinant biomedical proteins, genetic and molecular studies of restriction - modification phenomenon, developing of virus diagnostics, epigenetic study of small RNA, drug design and synthesis, bioinformatics.

Contents

This is the last time that I have the task of writing the preface to the Institute of Biotechnology Biennial Report. After 11 years in the post of the Institute Director, I retired at the end of June 2007. Looking back at Institute developments over this period, I am glad to see real progress in the Institute's striving for high quality of research. The Institute is distinctive by significant publications in well-known international journals and successful participation in international research programs. The Contents of this and previous Reports well reflect the new research results of the Institute's team.

There is no news the Institute will meet new challenges in the near future. Lithuanian society and public institutions expect the Institute will yield major input in the development of high technologies and novel products. The Institute always harmonized basic and applied research and I am sure that my colleagues have the capacities and creativity to address these big problems.

I wish my successor Prof. Kęstutis Sasnauskas all the best for the future and look forward to seeing the Institute tackle the problems that are topical both for pure knowledge and local biotech industry. I will use the vacant hours of retirement to assist Lithuanian Biotechnology Association in spreading its activities in all fields important for research and business biotechnologists.

I want to thank all my co-workers for their support, understanding and spirit of collaboration.

Algimantas Pauliukonis, Ph.D., Dr. habil.

In late June of 2007 after the election by the Institute's Council I took the leadership of the Institute of Biotechnology (IBT). The Institute harbors the outstanding research groups in diverse subjects of modern biotechnology and molecular biology, demonstrated by the numerous publications in the journals with high impact factors and intense collaboration with such leading companies as Abcam, Santa Cruz Biotechnology, Johnson & Johnson, Euroimmun, Micromun, Microimmun and others.

Besides, IBT is also licensed to carry out education of Ph.D. students in Biochemistry and Biotechnology together with Vilnius University and Gediminas Technical University, respectively. Many universities and research centres in Europe, Canada, Australia and the USA are involved in carrying joint research and collaboration with IBT.

I am very proud of the Institute's success in competition for various international grants and projects. Over the last period six grants within the European Union 5th Framework programme, including the award of the Centre of Excellence in 2002, nine grants related to the EU 6th Framework programme, four Howard Hughes Medical Institute grants and many others have been bestowed. Implementation of the repatriation policy has become possible at IBT due to Marie Curie reintegration grants for the Lithuanian researchers. It allowed establish the Laboratory of Bioinformatics and Laboratory of Biothermodynamics and Drug Design at IBT. These projects enabled to strengthen and broaden interdisciplinary research at the Institute and achieve better integration of research efforts on the basis of improved partnership and preparation of Lithuanian researchers for the implementation of the policy of the European Community in the sector of biotechnology.

The new spin-off company Profarma emerged from IBT in June of 2007 and I am happy to announce this event. Since 1991 it is the fifth IBT spin-off company following the previous ones: Fermentas, Biofa (currently Sicor TEVA), Biocentras and BIOK.

The future goals of IBT are focused on:

- foundation of biotechnological excellence in Lithuania;
- seeking new knowledge and concepts in biology at the molecular level;
- allying efforts with universities in Lithuania for improving training of graduate students and post-doctoral fellows;
- further collaboration with other institutions in Lithuania, Europe, the United States, Australia and elsewhere;
- proclaiming biotechnology in Lithuania as innovative and modern branch of basic and applied research in the foreign scale.

I hope IBT will meet challenges of the forthcoming changes within 2007-2013 EU Structural Funds Programmes and become an outstanding member of the knowledge based society.

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In a bacterial cell, restriction endonucleases (often referred to as restriction enzymes) act as a primitive immune system, protecting the cell from the invasion of foreign DNA, as would occur when a virus attempted to infect a bacterial cell. Restriction enzymes recognize short nucleotide sequences usually 4-8 bp in length and cut phosphodiester bonds in DNA introducing a double strand break. The host bacterium protects its own DNA from cleavage by "labeling" it through methylation of cytosine or adenine residues within the specific sequences recognized by the restriction enzyme. This modification renders host DNA refractory from the cleavage by restriction endonuclease while foreign DNA which lacks protective "methyl-tags" is degraded. Restriction endonucleases provided molecular biologists with a new tool to study and manipulate DNA by enabling the generation of consistently sized DNA fragments. In this respect restriction enzymes act as molecular scissors for specifically cutting DNA.

In the Laboratory of Protein-DNA Interactions we focus our studies on structural and molecular mechanisms by which restriction enzymes achieve their function. Among the questions being asked are:

- How do the restriction enzymes recognize the particular DNA sequence?
- How do the sequence recognition and catalysis are coupled in the function of restriction enzymes?
- How Nature engineered different specificities of restriction enzymes?

Answers to these questions are being sought using X-ray crystal structure determination of restriction enzyme-DNA complexes, site-directed mutagenesis and biochemical studies to relate structure to function.

Projects

Nucleotide flipping by restriction enzymes

Many DNA modification and repair enzymes require access to DNA bases and therefore flip nucleotides. Restriction endonucleases hydrolyze the phosphodiester backbone within or in the vicinity of the target recognition site and do not require base extrusion for the sequence readout and catalysis. Restriction endonuclease Ecl18kI is specific for the sequence /CCNGG ("/" designates cleavage site) and cleaves it before the outer C to generate 5 nt 5'-overhangs. We have solved the crystal structure of the Ecl18kI-DNA complex at 1.7 Å resolution. It turned out that Ecl18kI reads directly only the CCGG sequence and skips the unspecified N nucleotides, flipping them out from the helix. The extruded bases are buried in pockets within the protein. Hence, Ecl18kI is the first example of a restriction endonuclease that flips nucleotides to achieve specificity for its recognition site. Sequence and structure conservation predict nucleotide flipping also for the complexes of PspGI and EcoRII restriction enzymes with their target DNAs (/CCWGG). Using 2-aminopurine as a fluorescence probe we provided the first direct evidence that EcoRII-C and PspGI, similarly to Ecl18kI, flip nucleotides in solution.

Structural link between restriction endonucleases MvaI and BcnI and MutH enzyme of the bacterial DNA repair machinery

It has long been known that most Type II restriction endonucleases share a conserved core fold and similar active-sites. The same core folding motif is also present in the MutH protein, a component of the bacterial DNA mismatch repair machinery. In contrast to most Type II restriction endonucleases, which assemble into functional dimers and catalyze double-strand breaks, MutH is a monomer and nicks hemimethylated DNA. Our biochemical and crystallographic studies demonstrate that the restriction enzymes BcnI and MvaI share many additional features with MutH-like proteins, but not with most other restriction endonucleases. The structurally similar monomers all recognize approximately symmetric target sequences asymmetrically. Differential sensitivities to slight substrate asymmetries, which could be altered by protein engineering, determine whether the enzymes catalyze only single-strand nicks or double-strand breaks.

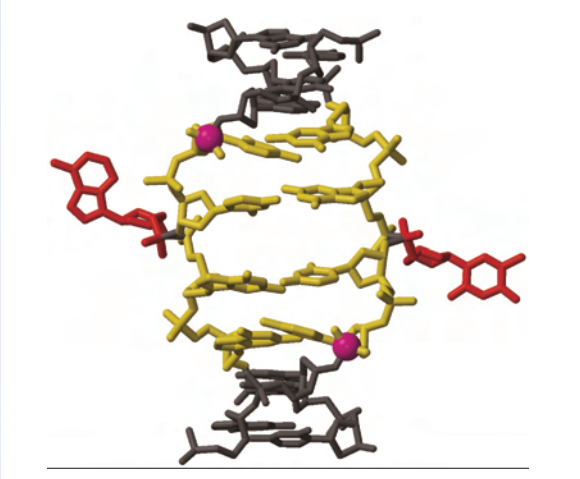


Fig. 1. *Central nucleotides are flipped out in the Ecl18kI-DNA complex*

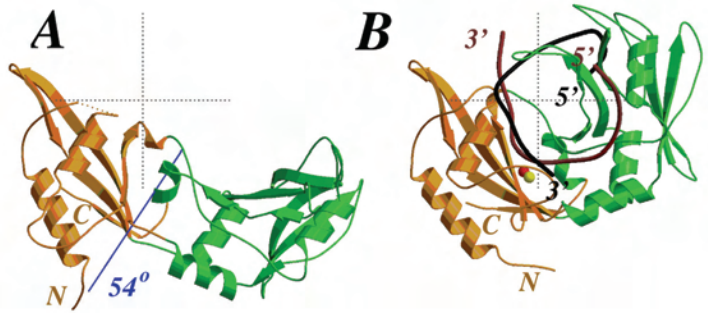


Fig. 2. *Apo- and DNA-bound structures of MvaI restriction enzyme*

Unexpected domain architecture of the rare-cutting restriction enzyme

Rare-cutting restriction enzymes are important tools in genome analysis. We have solved the crystal structure of SdaI restriction endonuclease, which is specific for the 8 bp sequence CCTGCA/GG ("/" designates the cleavage site). Unlike orthodox Type IIP enzymes, which are single domain proteins, the SdaI monomer is composed of two structural domains. The N–domain contains a classical winged helix-turn-helix (wHTH) DNA binding motif, while the C–domain shows a typical restriction endonuclease fold. The active site of SdaI is located within the C-domain and represents a variant of the canonical PD–(D/E)XK motif. SdaI determinants of sequence specificity are clustered on the recognition helix of the wHTH motif at the N–domain. The modular architecture of SdaI, wherein one domain mediates DNA binding while the other domain is predicted to catalyze hydrolysis, distinguishes SdaI from previously characterized restriction enzymes interacting with symmetric recognition sequences.

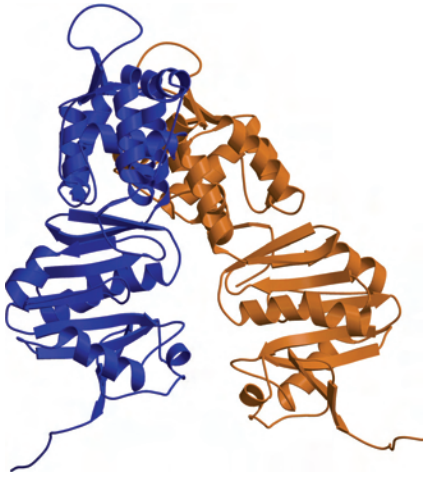
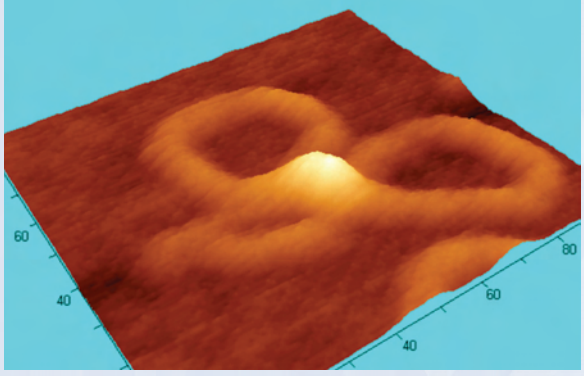


Fig. 3. *Domain architecture of SdaI restriction enzyme*

Restriction enzymes interacting with multiple recognition sites

The archetypal Type IIE restriction endonuclease EcoRII is a dimer that has a modular structure.
According to the current paradigm it simultaneously binds to two recognition sites but cleaves DNA at only one site per turnover: the other site acts as an allosteric locus, activating the enzyme to cleave DNA at the first. Structural and biochemical analysis of the EcoRII restriction enzyme suggests that it has three possible DNA binding interfaces enabling simultaneous binding of three recognition sites. To test if putative synapsis of three binding sites has any functional significance, we have studied EcoRII cleavage of plasmids containing a single, two and three recognition sites under both single turnover and steady state conditions. EcoRII displays distinct reaction patterns on different substrates: (i) it shows virtually no activity on a single site plasmid; (ii) it yields open-circular DNA form nicked at one strand as an obligatory intermediate acting on a two-site plasmid; (iii) it cleaves concertedly both DNA strands at a single site during a single turnover on a three site plasmid to yield linear DNA. Taken together the data indicate that EcoRII requires simultaneous binding of three rather than two recognition sites in cis to achieve concerted DNA cleavage at a single site. The AFM experiments provide direct experimental evidence that EcoRII synapses three recognition sites.

Fig. 4. *Synapsis of three recognition sites by EcoRII*



Restriction endonuclease Bse634I is a homotetramer arranged as a dimer of two primary dimers. Bse634I displays its maximum catalytic efficiency upon binding of two copies of cognate DNA, one per each primary dimer. The catalytic activity of Bse634I on a single DNA copy is down-regulated due to the cross-talking interactions between the primary dimers. The mechanism of signal propagation between the individual active sites of Bse634I remains unclear. Biochemical, mutational and kinetic analysis allowed us to identify two types of communication signals propagated through the dimer-dimer interface in the Bse634I tetramer: the inhibitory, or "stopper" and the activating, or "sync" signal. We suggest that the interplay between the two signals determines the catalytic and regulatory properties of the Bse634I and mutant proteins.

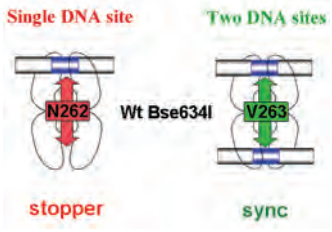


Fig. 5. *Communication signals propagated through the Bse634I interface*

Restriction enzymes with novel folds

The GIY-YIG nuclease domain was originally identified in homing endonucleases and enzymes involved in DNA repair and recombination. Many of the GIY-YIG family enzymes are functional as monomers. We show here that the Cfr42I restriction endonucle-

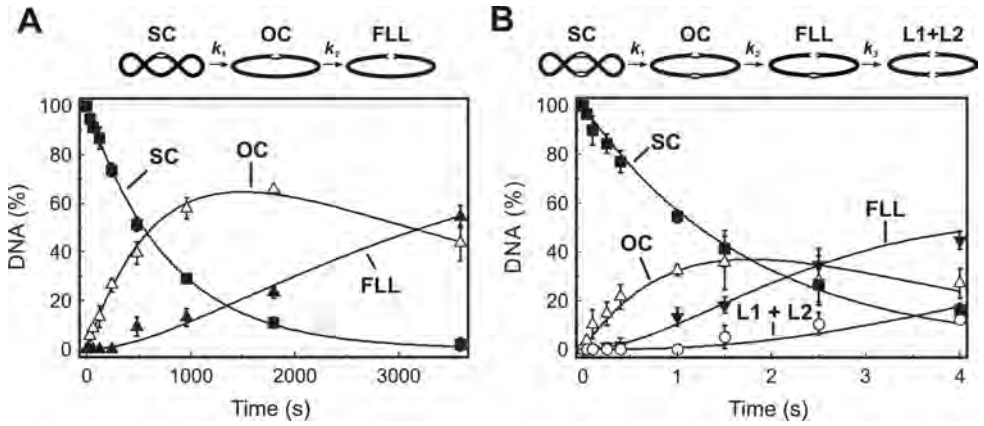


Fig. 6. *Cleavage of the 1- and 2-site plasmid DNA by Cfr42I*

ase which belongs to the GIY-YIG family and recognizes the symmetric sequence 5'-CCGC/GG-3' ('/' indicates the cleavage site) is a tetramer in solution. Moreover, biochemical and kinetic studies demonstrate that the Cfr42I tetramer is catalytically active only upon simultaneous binding of two copies of its recognition sequence. In that respect Cfr42I resembles the homotetrameric Type IIF restriction enzymes, like Bse634I, that belong to the distinct PD–(E/D)XK nuclease superfamily. To our knowledge, Cfr42I is the first tetrameric GIY-YIG family enzyme.

Type IIS restriction endonucleases recognize asymmetric DNA sequences and cleave both DNA strands at fixed positions downstream of the recognition site. REase BpuJI recognizes the asymmetric sequence 5'-CCCGT, however, it cuts at multiple sites in the vicinity of the target sequence. We show that BpuJI is a dimer, which has two DNA binding surfaces and displays optimal catalytic activity when bound to two recognition sites. Limited proteolysis experiments revealed that BpuJI is arranged of two domains: the N-terminal domain (NTD), which lacks catalytic activity but binds specifically to the recognition sequence as a monomer, and the C-terminal domain (CTD), which forms a dimer with non-specific nuclease activity. Fold recognition approach reveals that the CTD of BpuJI is structurally related to archaeal Holliday junction resolvases. We demonstrate that the isolated catalytic CTD of BpuJI possesses end-directed nuclease activity and preferentially cuts 3 nt from the 3'-terminus of blunt-ended DNA.

Novel reactions catalyzed by a restriction enzyme

Most restriction endonucleases use Mg^{2+} to hydrolyze phosphodiester bonds at specific DNA sites. We have shown that BfiI, a metal-independent restriction enzyme from the phospholipase D superfamily, catalyzes both DNA hydrolysis and transesterification reactions at its recognition site. In the presence of alcohols such as ethanol or glycerol, it attaches the alcohol covalently to the 5' terminus of the cleaved DNA. Under certain conditions, the terminal 3'-OH of one DNA strand can attack the target phosphodiester bond in the other strand to create a DNA hairpin. Transesterifica-

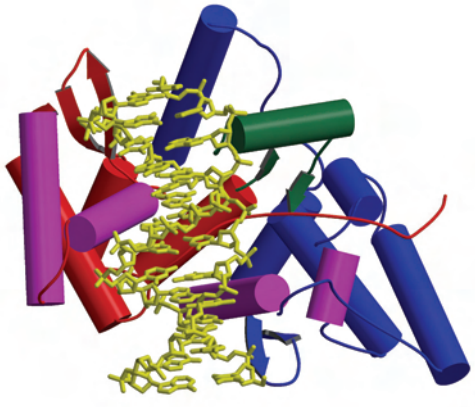


Fig. 7. *The BpuJI N terminal domain bound to DNA*

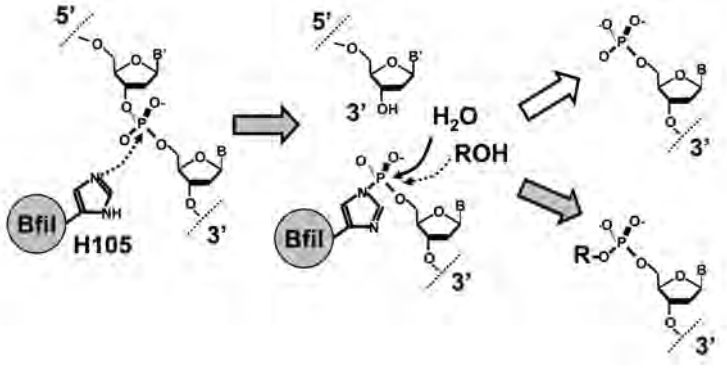


Fig. 8. *DNA transesterification and hydrolysis reaction catalysed by BfiI*

tion reactions on DNA with phosphorothioate linkages at the target bond proceed with retention of stereoconfiguration at the phosphorus, indicating, uniquely for a restriction enzyme, a two-step mechanism. We propose that BfiI first makes a covalent enzyme-DNA intermediate, and then it resolves it by a nucleophilic attack of water or an alcohol, to yield hydrolysis or transesterification products, respectively.

Novel molecular tools

The knowledge obtained during the structural and functional studies of restriction enzymes is used to engineer novel molecular tools for genome analysis and gene therapy. First strategy is based on the generation of restriction enzyme-Triple Helix Forming oligonucleotides (TFO) conjugates. It allows to address a particular recognition site of restriction enzyme within the genome through the triple helix formation. Second strategy employs the modular architecture of the Type IIS restriction enzymes. DNA binding and cleavage functions of Type IIS restriction enzyme BfiI are located on two physically separate domains. Moreover, in vitro studies of isolated DNA-binding domain of BfiI demonstrate that it binds to the target DNA as a monomer with an affinity comparable to that of wt enzyme. The latter finding makes DNA-binding domain of BfiI a challenging target for the exercise in protein design using phage display technology. Indeed, generation of the protein library of the DNA-binding domain and selection of variants with altered specificity followed by the fusion of the engineered domain to the catalytic module could generate restriction enzymes with novel specificities.

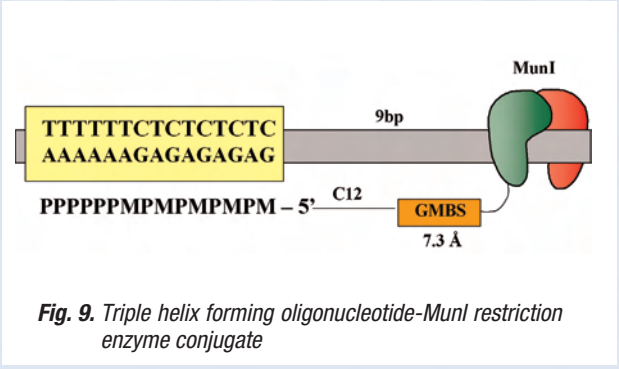
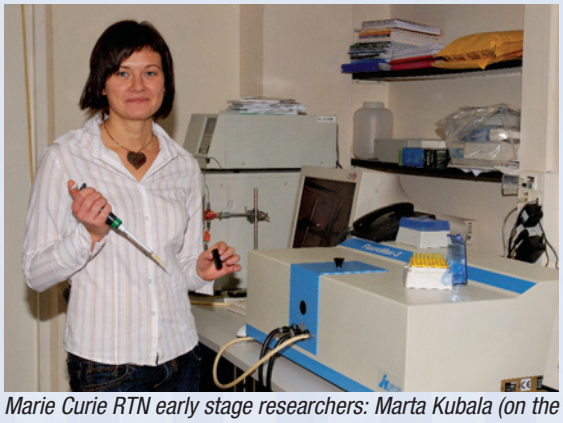
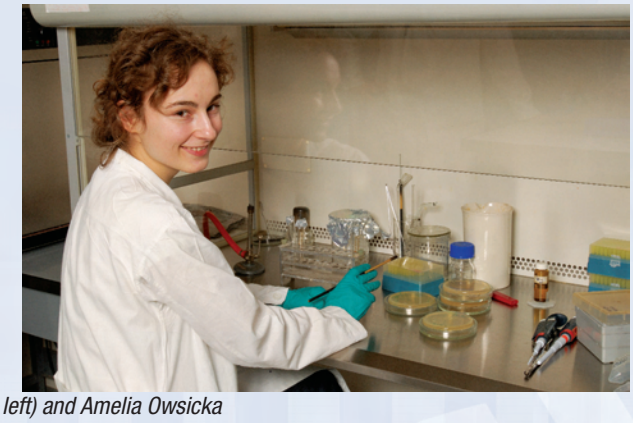
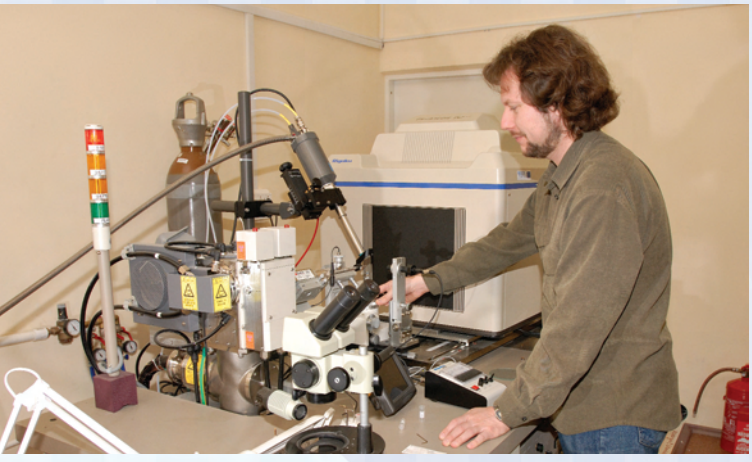


Fig. 9. *Triple helix forming oligonucleotide-MunI restriction enzyme conjugate*

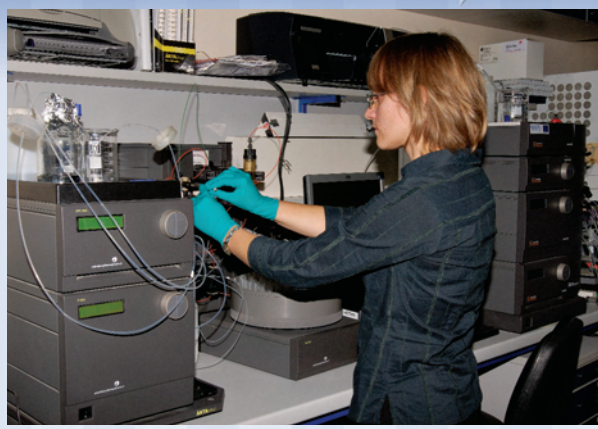




Marie Curie RTN early stage researchers: Marta Kubala (on the left) and Amelia Owsicka



Dr. S. Gražulis aligns crystal on the X-ray diffractometer



Dr. G. Tamulaitienė purifies proteins with AKTA FPLC system

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Publications 2006-2007

1. **Tamulaitis G.**, Mucke M., **Siksnys V.** Biochemical and mutational analysis of EcoRII functional domains reveals evolutionary links between restriction enzymes. FEBS Letters, 2006 (580):1665-71.
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5. **Zaremba M., Sasnauskas G.**, Urbanke C., **Siksnys V.** Allosteric Communication Network in the Tetrameric Restriction Endonuclease Bse634I. J. Mol. Biol., 2006 (363):800-12.
6. **Sasnauskas G.**, Connolly B.A., Halford S.E., and **Siksnys V.** Site-specific DNA transesterification catalyzed by a restriction enzyme. Proc. Natl. Acad. Sci. USA, 2007 (104):2115-20.
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AdoMet-dependent methyltransferases (MTases), which represent more than 3% of the proteins in the cell, catalyze the transfer of the methyl group from S-adenosyl-L-methionine (AdoMet) to N-, C-, O- or S-nucleophiles in DNA, RNA, proteins or small biomolecules. In DNA, enzymatic methylation of nucleobases serves to expand the information content of the genome in organisms ranging from bacteria to mammals. Postreplicative methylation is accomplished by DNA methyltransferases yielding 5-methylcytosine, N4-methylcytosine or N6-methyladenine. Genomic DNA methylation is a key epigenetic regulatory mechanism in high eukaryotes. DNA methylation profiles (occurrence of 5-methylcytosine) are highly variable across different genetic loci, cells and organisms, and are dependent on tissue, age, sex, diet, and other factors. Aberrant DNA methylation correlates with a number of pediatric syndromes and cancer, or predisposes individuals to various other human diseases. However, research into the epigenetic misregulation and its diagnostics is hampered by the lack of adequate analytical techniques. We aim to develop new approaches to genome-wide profiling of DNA methylation for epigenome studies and improved diagnostics.

Besides their diverse biological roles, DNA MTases are attractive models for studying structural aspects of DNA-protein interaction. Bacterial enzymes recognize an impressive variety (over 200) of short sequences in DNA. As shown first for the HhaI MTase, access to the target base, which is buried within the stacked double helix, is gained in a remarkably elegant manner: by rotating the nucleotide completely out of the DNA helix and into a concave catalytic pocket in the enzyme (Klimašauskas, S. et al., Cell 1994, 76: 357-369). This general mechanistic feature named “base-flipping” is shared by numerous other DNA repair and DNA modifying enzymes. Our laboratory has along standing interest in studies the mechanistic and structural aspects of DNA methylation using the HhaI methyltransferase (M.HhaI) from the bacterium Haemophilus haemolyticus as the paradigm model system. Although the methylation of biopolymers generally occurs in a highly specific manner, the naturally transferred methyl group has limited utility for practical applications. On the other hand, the ability of most MTases to catalyze highly specific covalent modifications of biopolymers makes them attractive molecular tools, provided that the transfer of larger chemical entities can be achieved. Our goal is to redesign the enzymatic methyltransferase reactions for targeted covalent deposition of desired functional or reporter groups onto biopolymer molecules such as DNA and RNA.

Kinetic and molecular mechanism of DNA methylation

Enzymatic DNA cytosine-5 methylation is a complex reaction that proceeds via multiple steps such as binding of cofactor AdoMet and substrate DNA, flipping of the target cytosine, conformational rearrangement of the mobile catalytic loop, activation of the target cytosine via formation of a transient covalent bond, methyl transfer and so on. We use mutagenesis, biochemical analysis, steady-state and transient kinetic analysis, fluorescence spectroscopy and x-ray diffraction to delineate the elementary steps on the reaction pathway of HhaI MTase (Klimašauskas S. et al., EMBO J. 1998, 17: 317-324; Serva S., et al., Nucleic Acids Res. 1998, 26: 3473-3479; Vilkaitis G. et al., J. Biol. Chem. 2001, 276: 20924-20934; Merkienė E., and Klimašauskas S. Nucleic Acids Res., 2005, 33: 307-315) and related enzymes (Vilkaitis G. et al., J. Biol. Chem. 2005, 280: 64-72; [6]) in collaborartion with groups of Prof. Shoji Tajima, Institute for Protein Research, Osaka University, Japan, Prof. Elmar Weinhold, Institute of Organic Chemistry, RWTH Aachen, Germany and Prof. Elizaveta Gromova, Moscow State University, Russia.

Rotation of a nucleotide out of the DNA helix (base flipping) is a mechanistic feature used by numerous modification and repair enzymes to gain access to their target bases buried in double-helical DNA. Despite of extensive studies of the HhaI cytosine-5 methyltransferase, initial events in the base flipping mechanism of this model enzyme remained elusive. Using mutagenesis, fluorescence spectroscopy and enzyme kinetics we demonstrated that the enzyme uses a protruding Gln237 residue for active opening the target C:G pair in DNA by a “push-and-bind” mechanism (Daujotytė, D. et al., Structure, 2004, 12: 1047-1055). In contrast to previous theoretical predictions, we have found that the crystalographically-inferred cross-helix hydrogen bond between the side chains of Gln237 and Ser87 plays no detectable role in target base flipping or the stabilization of reaction intermediates during catalysis (in preparation). In parallel, X-ray ‘snapshot pictures’ from a series of new crystal structures of the native and mutant HhaI methyltransferase variants with cognate or modified DNA substrates show the flipped out target base at various intermediate positions, suggesting a possible enzyme-assisted flipping pathway (in preparation).

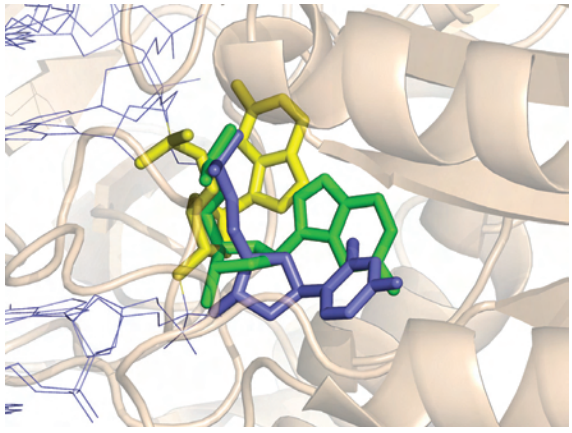


Fig. 1. *Overlay of co-crystal structures mutant M.HhaI-DNA complexes revealing distinct conformations of the target base along its flipping pathway (Daujotytė, Gražulis, Leinartaitė, Staševskij and Klimašauskas, in preparation).*

To obtain further insights into dynamics of the base flipping, an NMR structural study of the HhaI MTase-DNA system have been initiated in collaboration with Prof. Thomas Szyperski, SUNY Buffalo, NY, USA. We have engineered functional variants of the enzyme that show a significantly increased solubility and long term stability (Daujotytė, D. et al., Protein Engin. 2003, 16: 295-301). This now paves the way to detailed structural studies of this model cytosine-5 MTase and its interactions with the ligands employing NMR spectroscopy (in preparation).

DNA base flipping, first demonstrated in a crystal structure of the M.HhaI–DNA complex, now is known to be used by a wide variety of DNA enzymes. Fluorescence methods had been emplyoed for the determination and spectroscopic studies of base flipping in solution, and 2-aminopurine is often used as a fluorescent DNA base substitute (Holz B. et al., Nucleic Acids Res. 1998, 26: 1076-1083; Neely R.K. et al., Nucleic Acids Res., 2005, 33: 6953-6960). However, no simple and reliable technique for the detection of the natural nucleobase extrahelical cytosines is available. Chloracetaldehyde (CAA) is known to be used for chemical footprinting of single-stranded regions in nucleic acids. We have recently demonstrated the first application of CAA to detect individual extrahelical cytosines in the model M.HhaI-DNA complex, and then validated it by mapping unpaired extrahelical cytosines in unexplored systems including other DNA cytosine methyltransferases and restriction endonucleases (in preparation).

Targeted covalent modification and labeling of biopolymers

Our goal is to convert MTases into alkyltransferases for sequence-specific covalent modification of DNA and other biopolymers. Our strategy is based on designing novel synthetic analogues of the natural cofactor AdoMet. We have synthesized a series of model AdoMet analogs with sulfonium-bound extended side chains replacing the methyl group by direct chemical regioselective S-alkylation of AdoHcy. We demonstrated that allylic and propargylic side chains can be efficiently transferred by DNA MTases with high sequence- and base-specificity [1-2], which provides a novel enabling technique for sequence-specific covalent derivatization of DNA (reviewed in: Breindl, A. BioWorld Today 2005 16:1; Borman, S. Chem. Eng. News 2005, 83: 12; Mercer, A.C. & Burkart, M.D. Nature Chem. Biol. 2006, 2: 8-11). These cofactors are termed double-activated AdoMet analogs because the reactive carbon located between the sulfonium center and the unsaturated bond is activated for transfer by both adjacent groups [4, 9].

The aforementioned results demonstrate that methyltransferase-directed Transfer of Activated Groups (mTAG) is a convenient and robust technique suitable for routine laboratory use. As shown for restriction endonucleases, bulky groups can be deposited at specific sites to interfere with the action of DNA-modifying enzymes or DNA-binding proteins. Furthermore, functional groups could be appended to the side chains of the cofactors, sequence-specifically transferred to DNA and then modified with chemical entities in chemo-selective ligation reactions. Alternatively, a desired label could be directly attached to the side chain of the allylic and propargylic AdoMet analogs and sequence-specifically transferred to DNA. To this end, we have synthesized first AdoMet analogs with an extended propargylic side chain carrying a primary amino group [3], and synthesis of cofactors carrying a functional azide or thiol group in their extended side chain is in good progress.

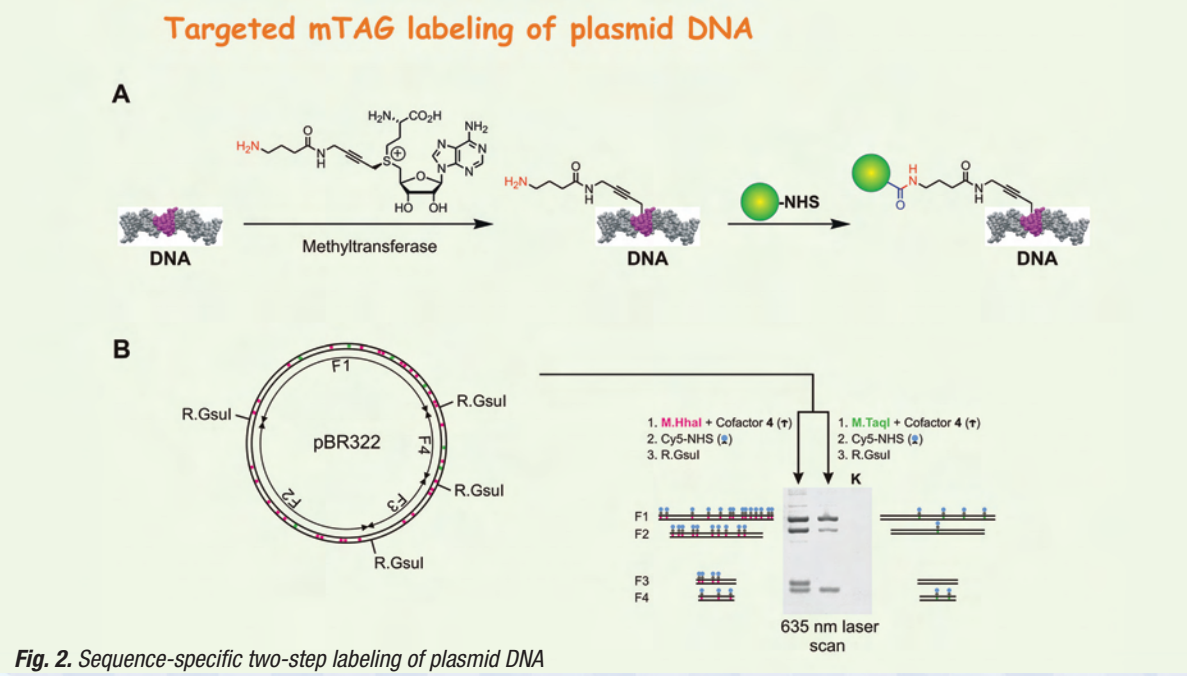


Fig. 2. *Sequence-specific two-step labeling of plasmid DNA*
A Principal scheme of a two-step mTAG labeling procedure. B. Sequence-specific two-step labeling of plasmid DNA with Cy5-NHS ester. pBR322 DNA was amino-modified with M.HhaI or M.TaqI in the presence of an extended AdoMet analog and then treated with a cyanine-5 N-hydroxysuccinimide ester (Cy5-NHS). Labeled DNA was fragmented with R.GsuI endonuclease to produce fragments F1–F4 and analyzed by agarose gel electrophoresis, followed by Cy5 fluorescence imaging with a 635 nm laser scanner. A DNA fragment (F3) containing no M.TaqI targets is not visible in the scan. K - unmodified pBR322 DNA treated with Cy5-NHS followed by R.GsuI fragmentation.

Using model DNA MTases along with their novel cofactors, we demonstrate that : i) mTAG approach can be used for efficient sequence-specific amino-functionalization of plasmid or bacteriophage DNA; ii) subsequent amine-specific chemoligations (with corresponding NHS esters) lead to covalent labeling of the natural DNA with reporter groups (biotin, fluorophores [3]); iii) labeled plasmid DNA can be used to efficiently transform E. coli cells (in preparation). These findings envision numerous applications of the new labeling technique in functional studies, DNA-based nanotechnologies and medical diagnostics (4). The REBASE database currently lists about 800 DNA MTases that recognize over 200 different DNA sequences spanning 2–8 base pairs, offering an unprecedented experimental control over sequence-specific manipulation of DNA with many potential applications ranging from probes for genetic screening technologies to molecular building blocks in DNA-based nanobiotechnology. Moreover, the newly developed cofactors should in principle be suitable for sequence-specific transfer of functional groups or other chemical entities to RNA and proteins using appropriate MTases as catalysts.

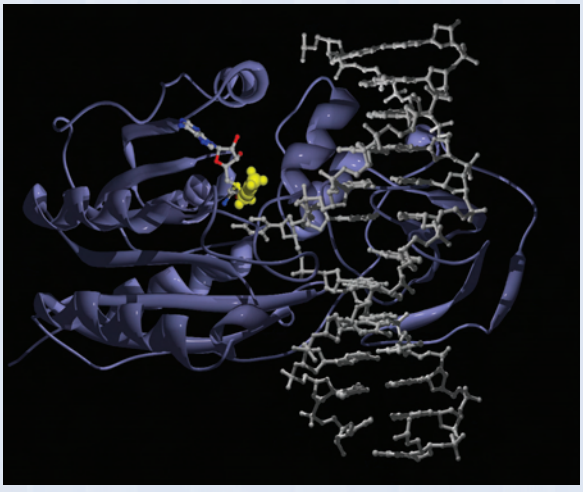


Fig. 3. *Rapid Transit: An alkynyl AdoMet analog is shown bound to a methyl-transferase (blue ribbon structure). The analog's extended group (yellow) is about to be transferred to DNA (gray) (from Chemical & Engineering News, December 5, 2005, Vol. 83, No 49, p. 12)*

Epigenome profiling

Genomic DNA methylation is a key epigenetic regulatory mechanism in high eukaryotes. DNA methylation profiles (occurrence of methylated cytosines) are highly variable across different genetic loci, cells and organisms, and are dependent on tissue, age, sex, diet, and other factors. Aberrant DNA methylation correlates with a number of pediatric syndromes and cancer, or predisposes individuals to various other human diseases. However, research into the epigenetic misregulation and its diagnostics is hampered by the lack of adequate analytical techniques. We therefore aim to develop new experimental approaches to genome-wide profiling of DNA methylation for epigenome studies and improved diagnostics. Our approach is based on selective mTAG labeling and enrichment of unmethylated CpG sites in the genome (note that premethylated target sites cannot be labeled [3]) followed by analysis of the enriched fractions on tiling microarrays (in collaboration with Prof. Art Petronis, CAMH, Toronto, Canada).

Functional analysis of small dsRNA methyltransferase HEN1

MicroRNAs and siRNAs are small non-protein-coding double-stranded RNA molecules that control gene activity in a homology-dependent manner - a process named RNA interference. Since their discovery in 1993, numerous microRNAs have been identified and recognized as important regulators of gene expression in both plants and animals. This number continues to rise with an astonishing speed. Currently, over 5400 molecules have been identified, and it is expected that the human genome encodes more than 2 % of small RNA genes which regulate expression one third of the human genes. Many microRNAs have well-defined developmental and tissue-specific expression pattern, but a great number of microRNAs and their roles are still unknown.

The biogenesis of miRNAs and siRNAs in plants differs from that in animals as it involves an additional methylation step catalyzed by the HEN1 methyltransferase. HEN1 from Arabidopsis catalyzes the transfer methyl groups from AdoMet onto the 2'OH group of the 3'-terminal nucleotide of small RNAs, like miRNA/miRNA* and siRNA/siRNA*. The methylation is imperative in the biogenesis of microRNA in Arabidopsis since microRNAs in hen1 mutants are reduced in abundance or are totally absent. number of molecular and biochemical approaches have been developed in our laboratory along with the group of Prof. Xuemei Chen at UC Riverside to examining the unique methyltransferase HEN1 [7]. Sequence analysis of this 942 residue protein predicts putative dsRNA-binding and La-like motifs near the N-terminus, and a C-terminal Rossman-fold methyltransferase region that includes conserved motifs characteristic of AdoMet-binding proteins. To determine the structural organization of HEN1, we have constructed a series of truncated His- and GST-tagged variants and compared their enzymatic activity and RNA-protein interaction in vitro using microRNA or siRNA duplexes. Our data show that the C-terminal domain alone, which includes the AdoMet-binding motifs, is sufficient for catalytic activity in vitro. However, different kinetic parameters of the truncated variant and the full length protein suggest that the N-terminal domain plays an important role in assembling a catalytically competent reaction complex. Further functional and structural analysis of the HEN1 MTase is currently underway.

Reconstitution of archaeal C/D box RNPs: towards synthetically programmable sequence-specific labeling of RNA

In archaea, C/D box small ribonucleoprotein complexes (sRNPs) direct site specific 2'-O methylation to numerous important sites in ribosomal and transfer RNA. The sRNPs are comprised of a C/D guide RNA which binds two copies of three proteins: L7Ae, Nop5p,

and aFib (the methyltransferase). Base pairing of guide sequences located upstream of either D or D' box to the RNA substrate targets the modifying enzyme to the site of methylation. A key feature of these RNA modification systems is that no constrains are placed on the substrate RNA beyond complementarity to the guide RNA sequence, and thus this system can be easily programmed to recognize any target RNA sequence. Combining the mTAG approach with sRNP MTases can be potentially used for programmable sequence-specific functionalization and labeling RNA, which would provide a new labeling technology with an unprecedented broad applicability. In collaboration with Dr. Beatrice Clouet d'Orval at Université Paul Sabatier, Toulouse, France, we have expressed the archaeal protein components, L7Ae individually and Nop5p-aFib as a complex, in E. coli, and purified them using His-tag affinity chromatography. The in vitro reconstituted Pyrococcus abyssi sRNP with sR47 guide RNA shows methylation activity with synthetic 15-mer substrates. A reliable analysis system for monitoring the modification of the target nucleotide by thin-layer chromatography (TLC) has been elaborated, which takes use of substrates with an internally 33P-labeled target nucleotide. Using this method the activity of the RNP and its mutant variants is being determined towards a range of synthetic AdoMet analogs containing sulfonium-bound extended side chains.

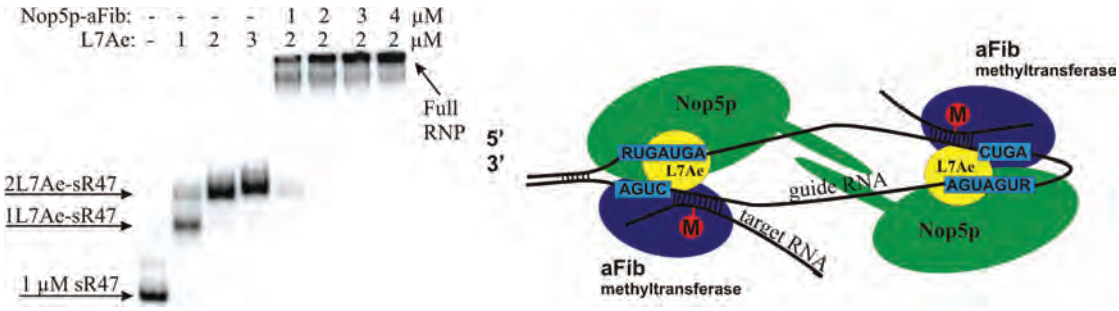
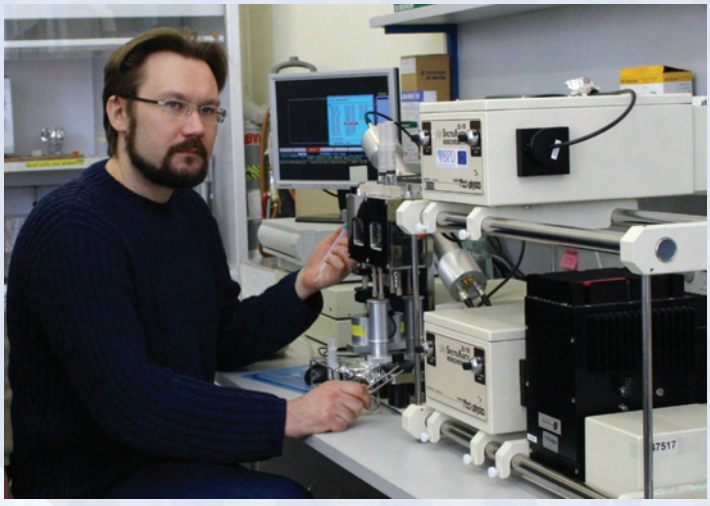


Fig. 4. *In vitro reconstitution of an archaeal C/D box sRNP complex. Left, a gel mobility shift assay revealing step-wise assembly of a full sRNP complex; right, a schematic structure of an archaeal sRNP, consisting of aFib, Nop5p and L7Ae proteins and sR47 guide RNA. Conserved C/D box sequences in the guide RNA are highlighted in blue boxes, transferred methylgroups (M) on two target RNAs are shown in red circles.*



Stopped-flow experiments performed by Dr. G .Vilkaitis

Organizers of the FEBS Workshop „DNA and RNA Modification Enzymes“ in Aussois, France (September 11-16, 2007): (from left) Saulius Klimašauskas (IBT, Vilnius), Henri Grosjean (Paris, France) and Janusz Bujnicki (Warsaw, Poland)

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Research in our laboratory is concentrated mainly toward the investigation of different aspects of the restriction-modification (RM) phenomenon, with emphasis on the practical applications of scientific results. We are focused on the sequence-structure-function relationships of the R-M components: restriction endonucleases (REases) and DNA methyltrans¬ferases. There are three main groups of restriction enzymes (types I, II, and III), which differ in enzyme composition, cofactor requirements, and mode of action. The best-studied are type II REases, which in general recognize specific DNA targets of 4–8 bp and cleave DNA at or close to these sequences. The exquisite accuracy of type II enzymes has made them indispensable tools for DNA manipulations. Although almost 3,700 type II REases with 262 different specificities have been characterized to date, there still is a demand for enzymes recognizing new DNA targets. To date, numerous attempts have been made worldwide to understand the mode of DNA recognition and to change the specificity of REases, the enzymes of high commercial value. This resulted in more than 15 high-quality X-ray structures of different REases. Nevertheless, information gained in this way appeared to be insufficient to rationally engineer restriction enzymes with new specificities. Recently, we have developed a technology called methylation activity-based selection for the isolation of REases with novel specificities. This technology allowed us to isolate, for the first time in the world, the REase Eco57MI with a new specificity. Through the last two years researchers in our laboratory demonstrated how to change specificity of Type IIB restriction endonucleases by swapping different target recognition domains (TRD) responsible for partial recognition of bipartite recognition sequence.

Exploring type IIB restriction endonucleases for altering DNA recognition specificities

During the past two decades, numerous efforts have been undertaken to engineer type II REases with altered specificities. Both rational protein design and random mutagenesis, followed by various selection procedures, have been tried, and several mutant enzymes with some preference for cleavage of altered DNA targets were isolated. However, projects concerned with orthodox type II REases so far have been largely unsuccessful mainly for two reasons: (i) difficulty of dealing with the observed tight coupling between DNA recognition and cleavage and (ii) absence of an efficient system for selecting enzymes with changed specificities. In this regard, unorthodox type II enzymes, such as the type IIG REase Eco57I (also investigated in our laboratory), have shown more promise. Type IIG enzymes combine the catalytic centers of endonuclease and methyltransferase in one polypeptide chain, and the ability of Eco57I to methylate recognized DNA targets has been applied to isolate mutants having previously undescribed specificity.

The discovery of AloI-like REases, classified as type IIB enzymes, opened up new opportunities for the engineering of type II REases with altered specificities. AloI-like REases are large polypeptides having both DNA endonuclease and methyltransferase activities (Fig.1). This group of enzymes recognize bipartite DNA targets and cleave DNA on both sides of recognition sequences. It turned out that C-terminal regions of AloI-like REases share sequence similarity with specificity (HsdS) subunits of some type I R-M systems. Based on this observation, we predicted that C-terminal regions of AloI-like REases, just like HsdS subunits, have two TRDs, each recognizing an individual part of the bipartite DNA target. By engineering active hybrids with swapped proximal TRDs among AloI, PpiI, and TstI, we have demonstrated a straightforward approach to generate type II REases of predictable specificity.

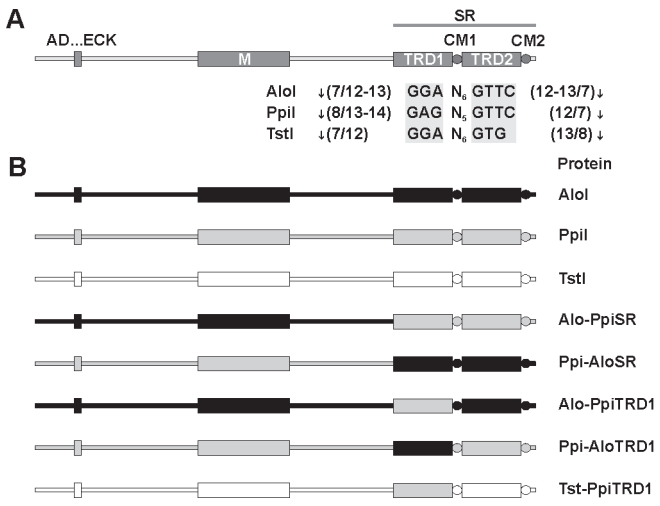

Fig. 1. *Schematic representation of progenitor enzymes AloI, PpiI, TstI, and their hybrids. (A) The structure–function organization and DNA recognition sequences of AloI, PpiI, and TstI R-M systems. (B) Progenitor enzymes and hybrids constructed in this work. AD... ECK, catalytic motif of endonucleolytic activity; M, protein region encompassing DNA methyltransferase motifs; CM1 and CM2, conserved sequence motifs. Specific components of DNA targets predicted to be recognized by TRD1 and TRD2 are depicted in rectangles; cleavage positions are indicated by arrows.*

Table 1. Specific activities of AloI, PpiI, TstI, and hybrid REases

Enzyme	Recognition sequence*	Specific activity†
AloI	↓(7/12–13)GGAN6GTTC(12–13/7)↓	4,700
PpiI‡	↓(8/13–14)GAGN5GTTC(12/7)↓	18,600
TstI	↓(7/12)GGAN6GTG(13/8)↓	18,900
Alo-PpiSR	↓(8/13–14)GAGN5GTTC(12–13/7)↓	4,600
Alo-PpiTRD1	↓(8/13–14)GAGN5GTTC(12–13/7)↓	4,600
Ppi-AloSR	↓(7/12–13)GGAN6GTTC(12/7)↓	9,500
Ppi-AloTRD1	↓(7/12–13)GGAN6GTTC(12/7)↓	18,900
Tst-PpiTRD1-Gly1006Leu§	↓(8/14–15)GAGN5GTG(13–14/8)↓	<1,500

† Specific activity is given as units per milligram of enzyme. One unit is defined as the amount of enzyme required to cleave 1 μg of BamHI-linearized DNA of pSEAd-7 in 1 h at optimal temperature in a reaction volume of 50 μl.
‡ Cleavage positions may vary depending on the DNA environment.

In addition, we have shown that computational protein structure modeling and evaluation methods are effective means for both designing and improving functional properties of hybrid type IIB enzymes. The significant increase in Tst-PpiTRD1 activity resulting from a single amino acid substitution suggested by the computational analysis indicates both the accuracy of the generated protein models and the prospects for further improvements. It is worth mentioning that the domain-swapping technique is not limited only to TRD1 swaps, because TRD2 exchange between PpiI and TstI enzymes can also generate REase of changed specificity.

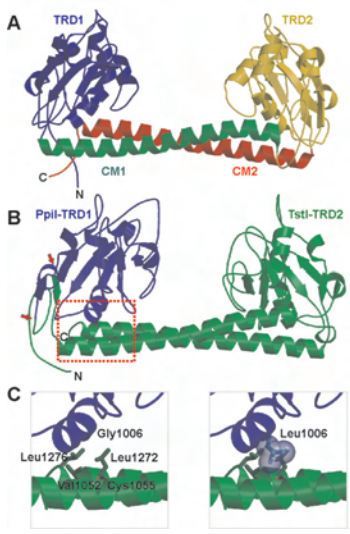

Fig. 2. *3D structures of the HsdS subunit of the type I R-M system from M. jannaschii (Protein Data Bank ID code 1YF2) and the computationally derived model of the SR of Tst-PpiTRD1. (A) The domain architecture of the HsdS subunit of the type I R-M system from M. jannaschii. Individual structural domains/motifs are colored from blue to red according to the progression of the polypeptide chain from the N to the C terminus. TRD1, TRD2, TRDs; CM1, CM2, conserved sequence motifs. (B) A model of the SR of Tst-PpiTRD1. TRD1 of PpiI is colored blue, the TstI moiety is shown in green. Red arrows indicate swapping points. A segment of the SR of TstI-PpiTRD1 enlarged in C is marked by a dotted square. (C) A close up view of the interface between TRD1 of Ppi and the conserved helices of TstI. The initial Tst-PpiTRD1 construct is on the left, and the Gly1006Leu mutant is on the right. Several side chains at the interface are shown as sticks with space-filling contour added for Leu-1006.*

Currently, there are 18 type IIB REases in REBASE, providing 21 unique TRDs. Theoretically, all these TRDs could be used in domain-swapping experiments. The sum of their combinations (>400), where each combination represents different DNA recognition specificity, exceeds the number of specificities (262) of type II enzymes known today. Obviously, not all of the TRD combinations might produce functionally active enzymes. Nonetheless, based on the results, we believe that the approach of combinatorial reassortment of TRDs of type IIB REases has the potential to greatly expand the list of available REase specificities. In addition, the potential pool of TRDs for constructing type IIB REases perhaps could be enlarged by borrowing some of these domains from HsdS subunits of type I R-M systems.

Identification of colicin-like catalytic HNH motif in type IIS restriction endonucleases

Type II restriction endonucleases (REases) cleave palindromic or non-palindromic short sequences of double-stranded DNA, and generally require Mg^{2+} ions for catalysis. Due to high sequence specificity and relatively simple structure Type II restriction endonucleases provide excellent model systems for studying protein-DNA interactions and evolutionary relationship among different protein groups.

Comparison of available crystal structures of REases suggests the presence of similar three-dimensional fold, termed the catalytic PD-D/ExK motif, pointing to a presumable evolution of these enzymes from a common ancestor. However, soon after the compelling hypothesis of relationship between all Type II enzymes, new studies have emerged that demonstrate convincingly that some REases are evolutionary and structurally unrelated to the PD-D/ExK superfamily. The MnlI REase is the first Type IIS enzyme characterized that employs a colicin-like catalytic HNH motif for cleavage of DNA substrates. A two-domain structure of MnlI has been identified by limited proteolysis, a common characteristic of the Type IIS group of REases. This feature makes them excellent targets for the study of a presumable evolutionary route of these enzymes through the exchange of structural and functional modules. An N-terminal domain of the MnlI enzyme mediates the sequence-specific interaction with DNA, whereas a C-terminal domain resembles non-specific bacterial colicin nucleases ColE7 and ColE9 in its requirement for alkaline earth as well as transition metal ions for double- and single-stranded DNA cleavage activities. The results indicate that the presumable fusion of the non-specific HNH-type nuclease to the MnlI-specific DNA binding domain had transformed MnlI REase into a Mg^{2+}-, Ni^{2+}-, Co^{2+}-, Mn^{2+}-, Zn^{2+}-, Ca^{2+}-dependent sequence-specific enzyme. Nevertheless, MnlI retains a residual single-stranded DNA cleavage activity controlled by its colicin-like nuclease domain. Cleavage of double- and single-stranded DNA in the presence of different metal ions is unparalleled among restriction endonucleases characterized to date.

Another type IIS restriction endonuclease Eco31I is a 'short-distance cutter', which cleaves DNA strands close to its recognition sequence, 5 -GGTCTC(1/5). Previously, it has been proposed that related endonucleases recognizing a common sequence core GTCTC possess two active sites for cleavage of both strands in the DNA substrate. Using bioinformatic analysis we identified a short region of homology between Eco31I and HNH nucleases. Three-dimensional model of the putative catalytic domain was constructed and validated by random and site-specific mutagenesis. The restriction mechanism of Eco31I was suggested by analogy to the mechanisms of phage T4 endonuclease VII and homing en-

donuclease I-PpoI. We proposed that residues D311 and N334 coordinate the cofactor, H312 acts as a general base activating water molecule for the nucleophilic attack, and K337 together with R340 and D345 are located in close proximity to the active center and are essential for correct folding of catalytic motif. We also predicted that the Eco31I catalytic domain contains a putative Zn-binding site, which is essential for its structural integrity. Our results suggested the HNH-like active site is involved in the cleavage of both strands in the DNA substrate. Thus, our data argue against the earlier prediction and indicate the presence of a single conserved active site in Type IIS restriction endonucleases that recognize common sequence core GTCTC.

Collaboration

Bujnicki J.M., International Institute of Molecular and Cell Biology, Poland

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Lithuanian State Science and Studies Foundation

Contracts

UAB Fermentas

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Hamster polyomavirus-derived virus-like particles as a promising universal carrier for vaccine development and hybridoma technology

The use of virus-like particles (VLPs) for vaccine development was stimulated by the successful introduction of the first human recombinant HBV vaccine based on yeast-expressed HBsAg-derived VLPs. VLPs can be generated by heterologous expression of viral capsid and envelope proteins and their subsequent spontaneous self-assembly in vivo or in vitro. VLPs mimick infectious viruses in their structural and immunological features but are non-infectious and highly safe because of the lack of a viral genome. Due to the repetitive antigenic structure, VLPs are highly immunogenic making them promising vaccine candidates against different pathogens.

The hamster polyomavirus (HaPyV) major capsid protein VP1 belongs to a family of polyomavirus VP1 proteins containing highly conserved structure motifs and functional domains. HaPyV VP1-derived VLPs offer a large panel of advantages making them a promising platform for vaccine development. These VLPs have been used as carriers for a variety of different foreign peptides, protein segments and entire proteins of different origin including virus- and cancer associated. A major reason for generation and using of chimeric VLPs is to transfer the intrinsic strong immunogenicity of the VLP carrier to per se low immunogenic peptide sequences. This was evidenced for a MUC1 peptide presented on HaPyV-derived VLPs; whereas the MUC1 peptide complexed to BSA did not induce a specific antibody response, chimeric HaPyV-VP1 VLPs harboring two copies of the peptide induce MUC1-specific antibodies (Zvirbliene et al., 2006a, b).

The localization of potential insertion sites for foreign peptide sequences is essential for the generation of chimeric VLPs as well as evaluation of foreign peptide size limits that can be inserted. In fact, fusion of longer or multiple copies of peptides/epitopes to VLPs usually introduces structural difficulties in protein folding and VLP formation. Comparative studies using defined model epitopes inserted into different positions of the HaPyV VP1-derived VLP carrier have demonstrated that the insertion site in the carrier determines the surface exposure, antigenicity and immunogenicity of the inserted foreign epitope on VLPs (Gedvilaite et al., 2006a; Aleksaite & Gedvilaite, 2006; Lawatscheck et al., 2007). These investigations demonstrated that insertion site #1 (between aa positions 80 – 89) and #4 (between aa positions 288 – 295) of HaPyV-VP1 are superior in terms of insertion capacity and induction of a B-cell immunity. The large insertion capacity of site #4 was confirmed by the generation of chimeric VLPs with an eGFP insertion (Gedvilaite et al., 2006a). We studied also the possibility to use for presentation of foreign protein segments the simultaneous insertion of the same or different foreign peptides into the same VP1 molecule (Lawatscheck et al., 2007; Aleksaite & Gedvilaite, 2006). A limitation of the insertion capacity of HaPyV-VP1 was observed for VP1 fusion proteins harboring foreign insertions at four different sites (Lawatscheck et al., 2007). The potential influence of a flexible linker insertion on both sides of foreign nonamer peptides (few CTL epitopes of human tumor-associated antigens) on the assembly of VLPs was also studied (Lawatscheck et al., 2007; Aleksaite & Gedvilaite, 2006). The insertion of a flexible GSSG linker seems to improve the immunogenicity of a HaPyV-VP1 harboring two insertions of a CEA epitope, but not of the corresponding VLPs harboring a single insert. Chimeric HaPyV-derived VLPs were demonstrated to be highly immunogenic, even when applied without adjuvant, especially chimeric HaPyV-VLPs harbouring a CEA epitope at insertion site #1 which induced a long lasting B-cell immunity in mice (Lawatscheck et al., 2007).

In addition to the humoral immunity, we have demonstrated that HaPyV VP1 VLPs were able to induce a T-cell immune response. Yeast-expressed VP1-VLPs originating from rodent, primate and human polyomaviruses were tested for their interaction with human monocyte-derived dendritic cells and murine spleen cell-derived dendritic cells and the induction of an in vitro T-cell response. HaPyV- and MPyV-derived VLPs were found to induce maturation of the dendritic cells as evidenced by increased levels of surface maturation markers and a reduced uptake of FITC dextran and Lucifer Yellow. Moreover, the dendritic cells stimulated with these VLPs produced interleukin-12 and stimulated CD8-positive T-cell responses in vitro (Gedvilaite et al., 2006a; Samonskyte et al., 2006).

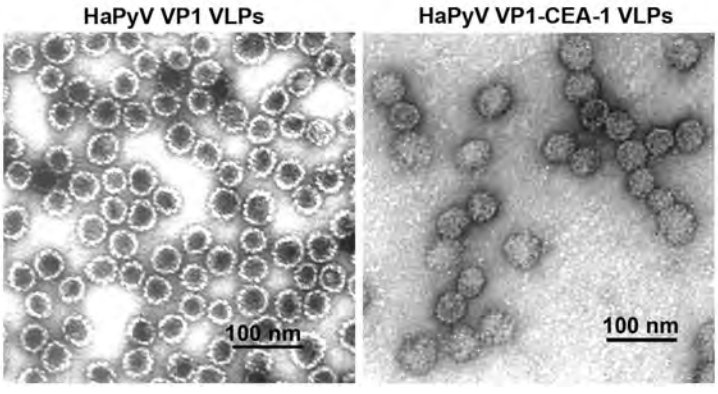

Fig. 1. *EM of virus-like particles (VLPs)*

A major drawback for the application of chimeric VLPs as vaccines in humans may represent a pre-existing immunity directed against the VLP carrier as the majority of the human population world-wide is persistently infected by the human polyomaviruses JCPyV and BKPyV. Few different strategies may solve the problems associated with a carrier-specific pre-existing immunity. We observed that the insertion of larger foreign protein segments into the VLP carrier demonstrated the reduction the intrinsic antigenicity and immunogenicity of the carrier itself. The level of reduction is related to the size of the foreign insertion; i.e. the larger the insert the lower the remaining VP1 antigenicity and immunogenicity. The generation of hantavirus nucleocapsid protein-specific mAbs by alternating immunization of mice with HaPyV-VP1 VLPs harboring a 120 aa-long segment of the N protein at sites #1 or #4, may underline the possibility to overcome the potential inhibitory effect of a pre-existing carrier-specific immunity on the antibody response against the foreign insertion (Zvirbliene et al., 2006). On the other hand, deletion mutagenesis analysis on the carboxy-terminal region of HaPyV-VP1 suggested that partial deletion within or truncation of this immunodominant region may also be a way to solve problems associated with the pre-existing immunity (Gedvilaite et al., 2006b)

An important basic research application of HaPyV VP1-derived VLPs was an investigation on the structure and assembly processes. VLPs were used to identify protein domains that are essential for VLP formation and responsible for shape and size determination using deletion mutagenesis approaches for HaPyV VP1 protein. Yeast-expressed truncation variants of HaPyV-VP1 lacking 35, 45 and 56 carboxy-terminal amino acid residues failed to form VLPs but those lacking 21, 69 and 79 amino acid (aa) residues at its carboxy-terminal region efficiently formed VLPs similar to those formed by the unmodified VP1 (diameter 40-45 nm). HaPyV-VP1 mutants with a single A336G aa exchange or internal deletions of aa 335 to aa 346 and aa 335 to aa 363 resulted in the formation of VLPs of a smaller size (diameter 20nm) (Gedvilaite et al., 2006b). Insertional mutagenesis using defined foreign insertions of different size and origin also allowed to confirm the structural flexibility of surface-exposed regions in VP1 (Zvirbliene et al., 2006)

Very successful was employment of HaPyV-VP1-derived chimeric VLPs as tools for hybridoma technology to generate monoclonal antibodies (mAbs) of desired specificity. Chimeric VLPs containing inserts of different size and origin were used as immunogens. The immunizations of mice were performed in Laboratory of Immunology and the results of this study are presented there.

In conclusion, HaPyV VP1-derived VLPs were useful tools for basic

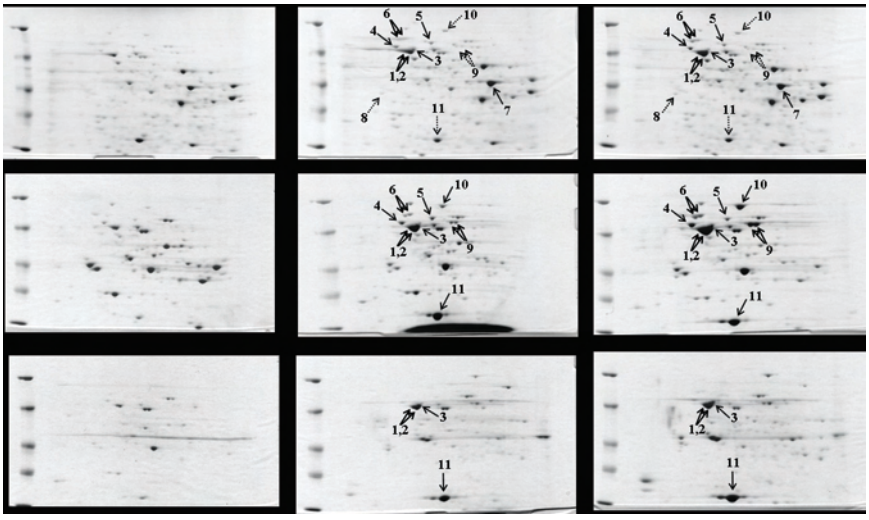

Figure 2. *2D gel electrophoresis of yeast proteins*

research applications, i.e. in structural and assembly studies and immunological investigations. HaPyV VP1-derived VLPs harboring foreign peptides or proteins may represent promising vaccines not only for viral infections but also for cancer and auto-immune diseases and are successfully employed for hybridoma technology.

Expression of glycoproteins of mumps and measles viruses in yeast

Proteomic approach was applied a to study molecular processes leading to formation of insoluble and inactive aggregates of recombinant mumps virus hemagglutinin-neuraminidase (MuHN) and measles virus hemagglutinin (MeH) in yeast Saccharomyces cerevisiae cells

Overexpression of cytoplasmic cell stress proteins Ssa1/2, Ssa4, Sse1, Hsc82, Hsp104, Sti1 and Sgt2 in response to MuHN and MeH synthesis indicated the presence of a stress response specific to accumulation of secretory protein precursors in the cytoplasm. Major cellular components of insoluble MuHN and MeH aggregates, directly interacting with recombinant viral proteins, appeared to be cytoplasmic heat shock proteins Ssa1/2p and Hsp26, the endoplasmic reticulum (ER) chaperone BiP/Kar2p was also identified. We may conclude the reason of inefficient virus surface glycoprotein expression in yeast lies on different protein maturation processes in mammalian and yeast cells, comprising translocation across the ER membrane and/or protein folding in the ER lumen. Now our lab is involved into the improvement of yeast expression systems for generation of active human virus surface glycoproteins.

Samples were taken from cells, expressing MuHN (central panel; B-H) or MeH (right panel; C-I) and from control cells, non-expressing these proteins (left panel; A-G). At the top (A-C) total protein lysates, in the middle (D-F) fractions of proteins, soluble at high salt concentration, and in the bottom panel (G-I) proteins, insoluble under native conditions are shown. Solid arrows in B and C indicate proteins, identified by MS directly from total yeast lysates, whereas dotted arrows point to the proteins, identified from soluble and insoluble fractions. M – MW markers.

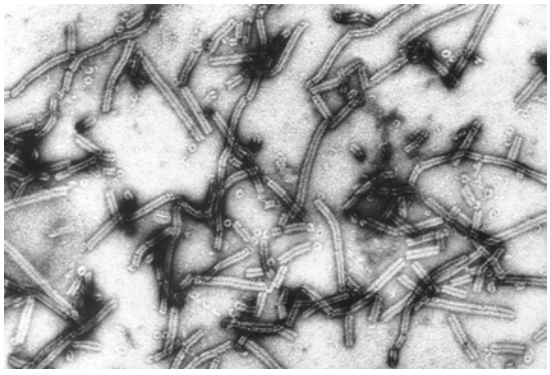

Figure 3. *EM of recombinant human parainfluenza virus 1 and 3 nucleocapsid proteins in yeast Saccharomyces cerevisiae (Juozapaitis M et al., 2007)*

Technology development of long-acting recombinant proteins of therapeutic value

The project is supported by the Lithuanian State Science and Studies Foundation grant N-07006

The new project in the area of therapeutic recombinant protein technologies has been started in the July of 2007. Innovative process was proposed for the production of long-acting (prolonged) form of human recombinant proteins such as granulocyte colony stimulating factor (GCSF) and different interferons (IFN). The essential element of this project reports to linear multimeric constructs of recombinant proteins that are produced by genetic engineering technique and expressed in E. coli. This study is continuation of previous research in the area of monomeric recombinant proteins of therapeutic value (Zaveckas et al., 2007).

Expected clearance rate of developed multimeric proteins may be

prolonged by more than several times exceeding clearance of homologous monomeric protein. New proteins are expected to compete with recombinant therapeutic proteins possessing prolonged activity in current markets. Linear multimers of the target protein obtained according developed process to highly purified state will be characterized by a set of analytical methods and tested for prolongation effect of their clearance on models in vitro and in vivo. Potential representative of protein multimers will be selected based on preclinical and PK/PD studies. The most potential protein candidates will go through further optimization of their lab-scale technology. It is expected that new technologies will be prepared for patent application procedure, with further licensing-in of the technology and implementation for industrial pharmaceutical production.

Hantavirus vaccine and diagnostic tools

The project was supported by the Lithuanian State Science and Studies Foundation grant T-83/07

Hantaviruses represent a separate genus Hantavirus of the family Bunyaviridae. They contain a tripartite RNA genome of negative polarity. The genome segments M (medium) and L (large) encode a glycoprotein precursor which is co-translationally processed into G1 and G2, and an RNA-dependent RNA polymerase. The S (small) genome segment codes for the nucleocapsid (N) protein. We have described the high-level yeast expression of authentic and amino-terminally His-tagged rN protein of PUUV (strain Vranica/Hällnäs) which are able to induce a protective immune response in bank voles, the natural host of PUUV (Dargevičiūtė et al., 2002).

High-level expression in yeast and purification of high yields of rN proteins of hantavirus species originating from Asia (HTNV) and different regions of Europe (PUUV strains Sotkamo and Kazan from Finland and Russia, respectively; DOBV-Slk from Slovakia and DOBV-Slo from Slovenia) was also performed. The rN proteins were characterized in terms of stability, nucleic acid and endotoxin contamination, antigenicity and immunogenicity (Razanskiene et al., 2004).

Hantavirus seroprevalence was investigated in Lithuania.

Recently we have started to investigate Hantavirus distribution in Lithuanian population (Sandmann et al., 2005). This work was extended with patients from dialysis centers. Serums of 218 patients from four dialysis centers of Kaunas district were tested for hantavirus specific antibodies by using the IgG antibody-capture ELISA. Antibodies against Dobrava/Hantaan and Puumala hantaviruses were found in 16 patients (seroprevalence 7.4%). Most of the sera were positive for Dobrava/Hantaan hantavirus (81%). Seroprevalence was significantly higher in older patients. In general the seroprevalence of dialysis patients is similar to previously estimated seroprevalence of patients from oncology center (Dargevicius et al. 2007).

New methods for hantavirus diagnostics

For the serological detection of human infections by hantaviruses relevant for Europe, we developed monoclonal antibody capture immunoglobulin G (IgG) and IgA enzyme-linked immunosorbent assays (ELISAs) based on yeast-expressed nucleocapsid proteins of Puumala and Dobrava hantaviruses. The specificities of the Puumala and Dobrava virus-specific IgA, and IgG ELISAs were found to be 100%. The sensitivities of these ELISAs were determined to be 100% with panels of characterized anti-Puumala or anti-Dobrava virus-positive human serum samples. In most cases, Puumala and Dobrava virus infections could be differentiated by ELISA reactivity alone, i.e., endpoint titration with homologous and heterologous antigens. (Meisel et.al. 2006)

Indirect and capture enzyme-linked immunosorbent assays (ELISAs) for detection of Hantaan virus (HTNV)-specific immunoglobulins G (IgG) and M (IgM) in human serum samples were developed on the basis of recombinant yeast-expressed nucleocapsid (N) protein of HTNV. The sensitivity of the indirect IgG and IgM ELISA tests was both 100% and the specificity of the indirect IgM and IgG ELISA test was 98% and 99%, respectively. The sensitivity and specificity of the capture IgM ELISA was 100% and 97%, respectively. The novel assays were found to detect HTNV-specific antibodies in acute phase sera from suspected HFRS patients in China. The results indicate that these novel ELISAs are suitable for the diagnosis of HTNV and for sero-epidemiological studies (Petraityte et al., 2007a).

For the detection of Hantaan virus in the oral fluid of humans, we have developed a monoclonal antibody-based capture enzyme-linked immunosorbent IgM assay (IgM capture ELISA) and indirect enzyme-linked immunosorbent IgG and IgM assays (indirect IgG and IgM ELISAs) for paired serum and oral fluid samples using the Saccharomyces cerevisiae yeast-expressed nucleocapsid protein of the Hantaan-Fojnica virus. The sensitivity and specificity of the oral fluid IgM capture ELISA in comparison with the results of the serum Hantaan virus IgM assay were 96.7% and of 94.9%, respectively. In conclusion, the IgM capture ELISA can be used with oral fluid instead of serum samples for the diagnosis of Hantaan virus infection (Petraityte et al., 2007b)

Biotechnological approaches to improve plant cold tolerance

This project is supported by the Lithuanian State Science and Studies Foundation grant N-07014.

In Lithuania, as in other temperate climate countries, plant cold hardiness is one of the major problems for farmers. Nearly every year, it is proven by large reimbursements, paid to farmers to recompense damages from unfavorable climate conditions. Insufficient cold hardiness of cultivated plants reduces the harvest and the harvest stability. Competitive abilities of farmers and attractiveness of agriculture business suffer.

Wild plants in nature have acquired numerous adaptations to survive in various climate conditions. The ability of some species to survive in extreme climate conditions shows that plant possibilities to adapt are considerable but in cultivated plants they could be improved. Some molecular aspects of cold resistance are elucidated in model plants as Arabidopsis thaliana. Our project is intended to use the knowledge of modern molecular biology to improve cold hardiness of plant species having industrial interest.

The main aim of this project is to create transgenic plants with improved cold resistance and cold acclimation. The object is to develop plant species having industrial value, such as winter rape, Miscanthus giganteus and horticultural plants.

Detection and elimination of viroids and phytoplasmas from horticultural crops used in industrial biotechnology

This project is supported by the Lithuanian State Science and Studies Foundation grant N-07010.

Rapidly evolving molecular biology provided new tools for plant phytopathology. As a consequence, the smallest known pathogens (viroids and mollicutes – phytoplasmas and spyroplasmas) had been discovered that received huge worldwide attention due to the economical damage done by them to the agriculturally valuable crops.

In Lithuania, before launching this project only few phytoplasmas had been found in pear, cherry, apple trees and strawberry plants while viroids had never been studied at all. Therefore, more comprehensive studies are needed to evaluate diversity of and damage being done by phytoplasmas and viroids in valuable plants in Lithuania.

This project is aimed at identification of the harmful viroid and phytoplasma species in Lithuania, evaluation of their disperse in horticultural crops used in industrial biotechnology and revealing the possibilities of growing viroid- and phytoplasma-free plants by applying thermo-, cryo-, and chemo-therapy in vitro. While running this project we plan to determine existence of viroids in Lithuania, to evaluate yet unknown disperse of mollicutes (phytoplasmas and acholeplasmas), to identify and characterize the detected pathogens by applying molecular techniques, to evaluate their genetic and biological properties and damage done, to collect necessary information about the detected pathogens and to develop molecular pathogen detection methods by designing new primer groups, to evaluate possibilities of horizontal DNA transfer within the phytoplasmas' genome and to evaluate the possibilities of eliminating viroids and phytoplasmas from the infected plants in vitro. In 2007 the gardens as well ecological plantations were inspected in the Southern (Alytus and Marijampolė regions), Central (Kaunas region), North-Western (Šiauliai region) and Eastern (Vilnius region) Lithuania, 93 plant samples were collected. Of them, 68 were examined by applying Return Polyacrylamide Gel Electrophoresis or PCR techniques to detect viroid RNAs and phytoplasmas respectively. The results obtained suggest that 5 trees (4 sweet cherry and 1 apple tree) presumably are viroid infected while phytoplasmas were found in the samples of 18 plants. Using AY1 strain of the 16SrI type phytoplasma new PCR primers were designed that allow accelerating detection of phytoplasmas. The plants being infected by phytoplasmas and viroids also suffer heavily from the other microorganisms (bacterias and fungi). This prevents creating a sterile plant culture in vitro.

Plant genetic engineering experiments performed by R.Vorobjovienė, M.Sc.

Prof. Robert E. Davis from Plant Sciences Institute (USA) at the Institute of Biotechnology in November 2007

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Micromun GmbH, Germany
Friederich-Loeffter-Institut, Germany
Euroimmun AG, Germany
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UAB Profarma, Lithuania
UAB Fermentas, Lithuania

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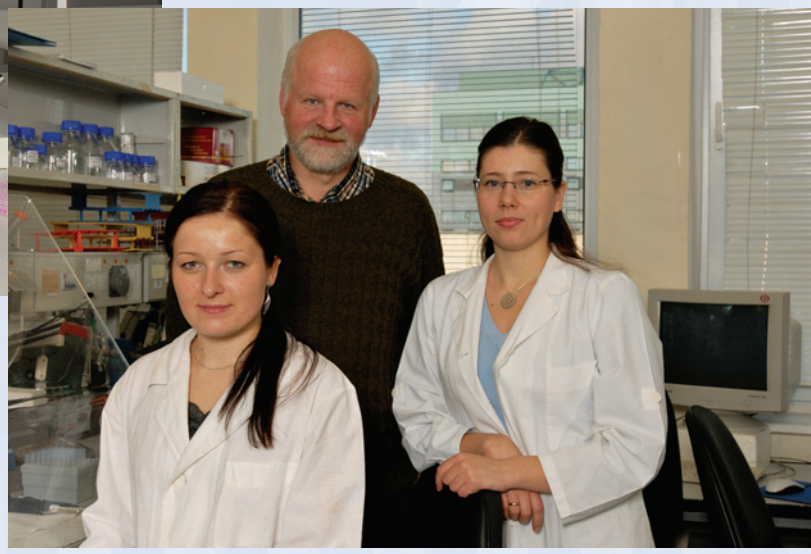
Patents

Zvirbliene A., **Gedvilaite A.**, Ulrich R., **Sasnauskas K.** A mean to produce monoclonal antibodies; hybridoma cell lines generated by this mean, and recombinant chimeric virus-like particles with inserted foreign protein fragments as immunogens to generate hybridomas by this mean. Patent No. 5414 (Lithuanian Patent Office, 25.04.2007).

Laboratory of Immunology and Cell Biology



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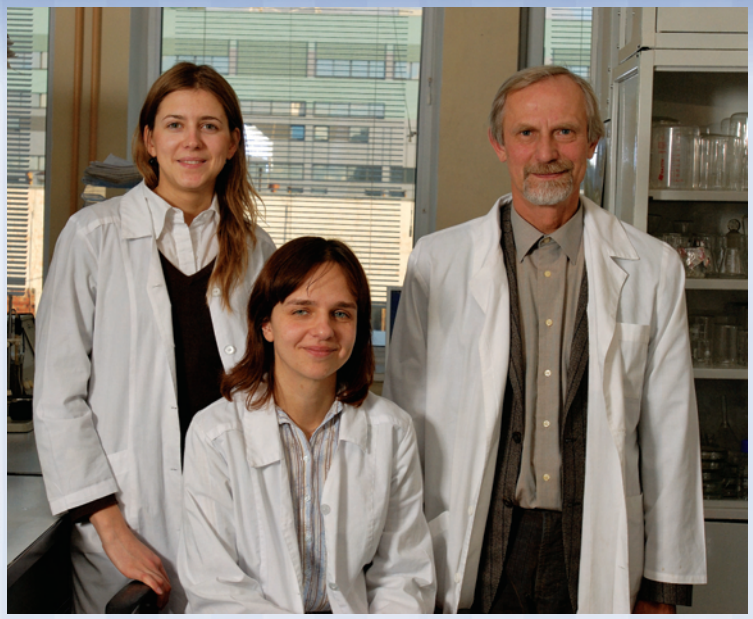


The Laboratory of Immunology and Cell Biology consists of three research groups. In 2006-2007, the research was focussed to the following topics: antigenicity studies of recombinant viral proteins and development of monoclonal antibodies (Dr. A.Žvirblienė), regulation of gene expression by alternative splicing (Dr. A.Kanopka), molecular epidemiology of tuberculosis (Dr. P.Stakėnas).

Antigenic characterisation of yeast-expressed viral proteins

This work was performed in collaboration with the Laboratory of Eukaryote Gene Engineering. It was aimed at evaluating the antigenic properties of yeast-expressed recombinant proteins that might be exploited as potential vaccines or diagnostic tools.

Using different immunochemical assays, the antigenic structure of yeast-expressed measles nucleocapsid (N) protein and lyssavirus N proteins was investigated. These recombinant antigens were expressed in the Laboratory of Eukaryote Gene Engineering.

The yeast-expressed measles N protein used in this study was self-assembled into nucleocapsid-like structures similar to that of native virus. To identify B-cell epitopes in measles N protein, we have employed monoclonal and polyclonal antibodies raised against recombinant N protein as well as human sera from measles-positive individuals. The localization of B-cell epitopes was studied using recombinant overlapping N protein fragments, PepScan analysis and competitive ELISA. The majority of monoclonal antibody epitopes were mapped within the C-terminus of N protein. Cross-inhibition studies with human sera demonstrated similar localization of B cell epitopes recognized by serum antibodies from naturally infected individuals, which reveals a clear antigenic similarity between recombinant and measles virus-derived N protein. The results of the current study are in agreement with previous structural studies of measles N protein showing the accessibility of the C-terminal domain on the surface of viral nucleocapsids. These findings may have important implications for the design of new recombinant measles vaccines and diagnostic reagents (Zvirbliene et al., 2007).

In Europe, three genotypes of the genus Lyssavirus, family Rhabdoviridae, are present: classical rabies virus (RABV, genotype 1), European bat lyssavirus type 1 (EBLV-1, genotype 5) and European bat lyssavirus type 2 (EBLV-2, genotype 6). The N proteins of RABV, EBLV-1 and EBLV-2 were expressed in yeast Saccharomyces cerevisiae and purified by density-gradient centrifugation. As demonstrated by electron microscopy, the purified N proteins were self-assembled to nucleocapsid-like structures. The antigenic structure of the N proteins was investigated for their reactivity with monoclonal antibodies (mAbs) directed against different lyssaviruses. The reactivity pattern of each mAb was virtually identical between immunofluorescence assay with virus-infected cells, and ELISA and dot blot assay using the corresponding recombinant N proteins. These observations lead us to conclude that yeast-expressed lyssavirus N proteins share antigenic properties with naturally expressed virus protein. These recombinant proteins have the potential for use as cost-effective antigenic components of serological assays for lyssaviruses. This could find application in the assessment of the response to rabies vaccination and a virus-specific screening antigen for the measurement of seroprevalence for active surveillance of EBLVs in European bats (Kucinskaite et al., 2007).

Generation of monoclonal antibodies of desired specificity using chimeric virus-like particles

The production of monoclonal antibodies (mAbs) directed against selected epitopes or peptides has important implications for functional and clinical studies. Such antibodies have become indispensable tools in biochemistry and molecular biology, including microarray-based proteomics. For the generation of anti-epitope antibodies, synthetic peptides are commonly used. Because of their size, peptides are usually not immunogenic and have therefore to be coupled to larger carrier proteins. An efficient immune response to the desired peptide can be achieved with a careful selection of the carrier by which the peptide can be displayed to the immune system. Synthetic peptides when coupled to carrier proteins such as bovine serum albumin, ovalbumin or keyhole limpet hemocyanin, usually elicit a strong humoral immune response. However, many factors influence the immunogenicity of the peptide, such as method of coupling, length of the sequence, its hydrophilicity, accessibility, mobility and protrusion. The problem that is encountered when preparing anti-peptide antibodies is whether the peptide sequence is displayed on the surface of the peptide-carrier conjugate to be accessible to B cells. It is well known that

the antigenic parts of a protein surface are located predominantly in loops and/or protruding regions.

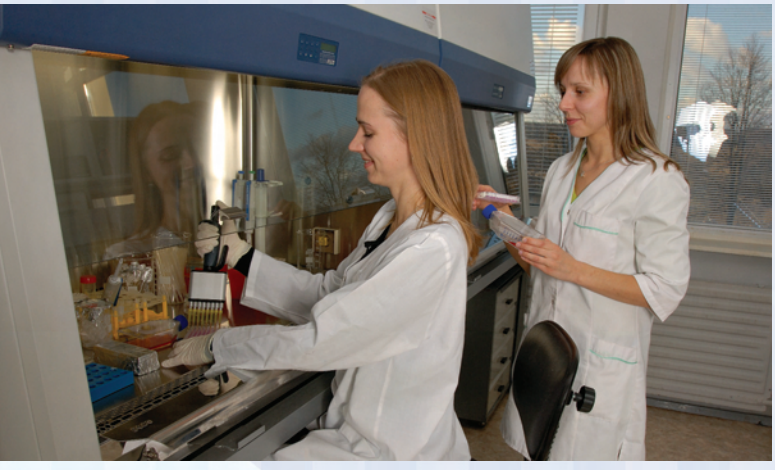
Protein engineering provides an opportunity to generate new immunogens with desired features. Viral structural proteins with their intrinsic capacity to self–assemble to highly-organized virus-like particles (VLPs) have been shown to possess high immunogenicity and have been exploited as potential vaccines. Previous studies demonstrated that insertions/fusions of foreign protein segments at certain sites of VLP carriers did not influence protein folding and assembly of chimeric VLPs.

In the Laboratory of Eukaryote Gene Engineering, chimeric VLPs representing major capsid protein VP1 of hamster polyomavirus (HaPyV) with inserted foreign sequences at certain surface-exposed regions were expressed in yeast Saccharomyces cerevisiae. These chimeric HaPyV-VP1 VLPs have been shown to induce in mice a strong antibody response against the inserts (Gedvilaite et al., 2000, 2004). Thus, the chimeric VLPs meet the requirements for a strong immunogen being able to activate both B cells recognizing the surface-located epitopes and T helper cells providing the necessary signals for Ig class switching and affinity maturation. We have employed chimeric VLPs harbouring foreign sequences of different size and origin to generate insert-specific mAbs. The length of inserts ranged from 6 to 280 amino acids. It was demonstrated that chimeric VLPs efficiently stimulated the production of IgG antibodies specific for the sequences/epitopes presented at surface-exposed regions. This approach was successfully used to generate mAbs against non-immunogenic protein sequences. Moreover, it was demonstrated that the insert-specific mAbs recognized native full-length proteins, which suggested the correct folding of the sequences displayed on VLPs. Our data confirm that the insertion of non-immunogenic epitopes into VLPs significantly increases their ability to induce a strong B cell response. Thus, chimeric VLPs represent efficient immunogens for hybridoma technology and provide a promising alternative to chemical coupling of synthetic peptides to carrier proteins (Zvirbliene et al., 2006).

Based on these investigations, the patent application was filed (PCT/EP2006/003420, WO2006/108658). The licensing agreement with UAB Fermentas was signed (20.12.2006).

This work was supported by the Lithuanian State Science and Studies Foundation (grants No. B-16/2006, G-06/07) and UAB Fermentas (grant No. 11/07).

Junior scientist I. Sezaitė and master student I. Kairytė perform immunization



PhD student I. Kučinskaitė and bioengineer R. Lasickienė work with hybridoma cells

Regulation of hypoxia-inducible factor HIF-3 expression via the alternative pre-mRNA splicing mechanism

Recent genome-wide analyses of alternative splicing indicate that up to 70% of human genes may have alternative splice forms, suggesting that alternative splicing together with various posttranslational modifications plays a major role in the production of proteome complexity. Splice-site selection under normal physiological conditions is regulated in the developmental stage in a tissue type-specific manner by changing the concentrations and the activity of splicing regulatory proteins.

Changes in splice-site selection have been observed in various types of cancer and may affect genes implicated in tumor progression and in susceptibility to cancer. Splicing defects can arise from inherited or somatic mutations in cis-acting regulatory elements (splice donor, acceptor and branch sites, and exonic and intronic splicing enhancers and silencers) or variations in the composition, concentration, localization, and activity of regulatory proteins. This may lead to altered efficiency of splice-site recognition, resulting in overexpression or down-regulation of certain splice variants, a switch in splice-site usage, or failure to recognize splice sites correctly, resulting in cancer-specific splice forms. At least in some cases, changes in splicing have been shown to play a functionally significant role in tumorigenesis, either by inactivating tumor suppressors or by gain of function of proteins promoting tumor development. Thus, the identification of cancer specific splice forms provides a novel source for the discovery of diagnostic or prognostic biomarkers and tumor antigens suitable as targets for therapeutic intervention.

Hypoxia has long been recognized as a common feature of solid tumors and a negative prognostic factor for response to treatment and survival of cancer patients. The discovery of hypoxia-inducible factor 1 (HIF-1), a molecular determinant of the response of mammalian cells to hypoxia, has led to the identification of a ''molecular target'' of hypoxia suitable for the development of cancer therapeutics. Immunohistochemical analyses using monoclonal antibodies revealed that HIF-a is overexpressed in the majority of human cancers, where it is associated with patient mortality and poor response to treatment. As it is established HIF-1 role in cancer biology is complex. HIF-1 inhibitors should be incorporated in combination strategies to effectively target multiple cellular components of the tumor microenvironment and redundant signaling pathways frequently deregulated in human cancer.

The role of other two HIFa subunits (HIF-2a and HIF-3a), in different tumor types, still remains to be defined.

A splice variant of HIF-3, inhibitory PAS domain protein (IPAS), inhibits the dimerization of HIF-1 and ARNT. This protein is translated by an alternatively spliced variant of mouse HIF-3 mRNA, which shares exons 2 – 6 with HIF-3, but which contains exons 1 and 7 different from those of the wild-type mRNA. Since all exons including exons 1 and 7 are present near the Hif 3 locus, this variant must be a spliced mRNA transcribed by the Hif 3 gene. IPAS protein contains a bHLH domain and a PAS domain, which are the common structures present in the HIF family. However, it has no transactivation function due to its lack of a transactivation domain, but does dominantly and negatively regulate HIF-mediated gene expression. IPAS expression in hepatoma cells selectively impairs the induction of hypoxia-inducible genes regulated by HIF-1 and results in retarded tumor growth and tumor vascular density in vivo. In mice, IPAS was selectively expressed in Purkinje cells of the cerebellum and in the corneal epithelium of the eye. Moreover, the expression of IPAS in the cornea correlates with low VEGF gene expression under hypoxic conditions.

We established that essential splicing factors SR proteins are involved in dependable from oxygen tension pre-mRNA splicing regulation.

The identification of selective HIF-1 inhibitors which will reprogram HIF-1 function would not only be useful for the potential therapeutic implications but also for their application as analytic tools.

This work was supported by the EU Framework 6 Programme (project Euroxy).

Molecular epidemiology of Mycobacterium tuberculosis in Lithuania

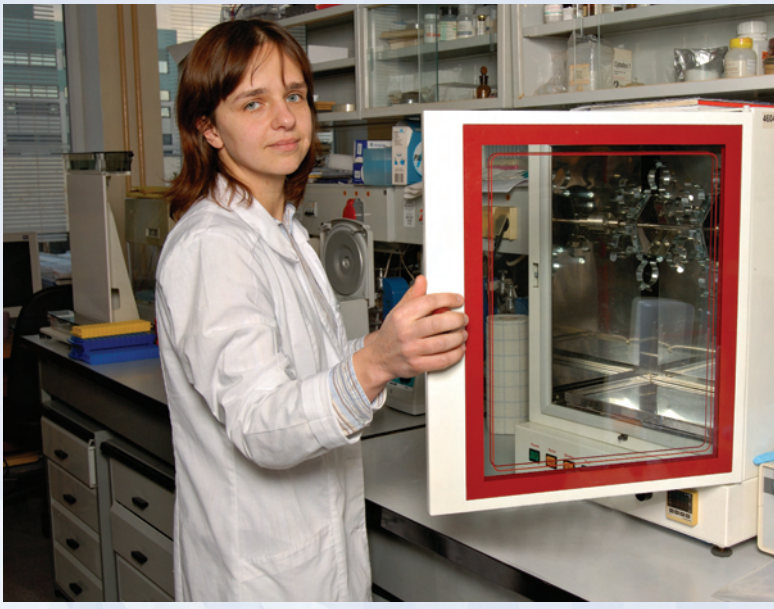
This work was performed in collaboration with the National Hospital of Tuberculosis and Infectious diseases (Vilnius, Lithuania).

Tuberculosis (TB) is a serious global health problem. An estimated one third of the world's population is infected with M. tuberculosis complex bacteria causing nine million of new cases and two million deaths annually. The most effectively tuberculosis is cured according to WHO recommended directly observed treatment short-course (DOTS) strategy. However, an improper management of tuberculosis has led to emergence of multidrug-resistance (MDR), defined as resistance to the two most powerful anti-tubercular drugs isoniazid and rifampin at least. According to DOTS-plus strategy, multidrug-resistant tuberculosis is treated by using the second-line antituberculosis drugs and in the developed countries between 60 and 80% of patients is cured. However, such treatment is far more expensive and in developing nations the majority of patients suffering from multidrug-resistant tuberculosis are condemned to die. Moreover, the average patient may infect a further 15 to 20 people. Therefore, it is of great importance to improve the understanding of the transmission of tuberculosis and the mechanisms of acquisition of drug resistance of M. tuberculosis.

Tuberculosis situation in Lithuania remains alarming. In year 2006, there were 2365 notified TB cases (notification rate 69.68 per 100 000 of population), spread of MDR TB was one of the highest in the world (9.4% of new and 46.6 of previously treated TB cases were MDR). In this context, there are strong reasons for increased efforts, including scientific research efforts, to counteract the threatening situation. Therefore, the research group from the Laboratory of Immunology and Cell Biology have continued integration of molecular epidemiology research into National strategy for control of drug-resistant-tuberculosis with the aims to prognosticate possible changes in the population of M. tuberculosis and to evaluate the efficiency of implementation of the Strategy as well.

The research of the last two years was focused on characterisation in detail population of M. tuberculosis including genetic determinants of drug resistance which has circulated in Lithuania.

In total, 430 M. tuberculosis strains recovered from different patients suffering from tuberculosis were investigated by internationally standardized restriction fragment length polymorphism (RFLP) typing using the insertion element IS6110 as a probe. Of them 45% were MDR that comprised more than a quarter of all



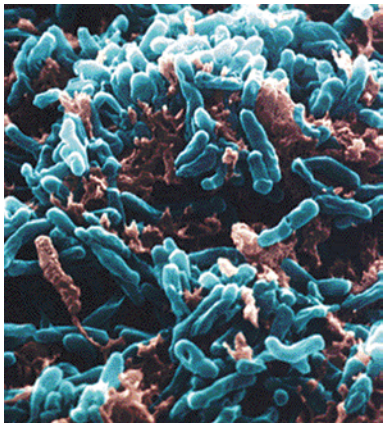
Junior scientist D. Bakonytė, M.Sc. performs DNA hybridization

MDR TB cases. Genotyping analysis revealed further expansion of four large groups of highly virulent and genetically related strains identified by us previously. The strains of these groups have caused more than 90% of all MDR TB cases. The proportion of clustered strains during three year did not change significantly indicating on stabilisation of TB situation in Lithuania. However, detail epidemiological analysis has showed, that the chains of transmission the most prevalent strains including MDR ones have not been broken. Thus, an essential improvement of TB situation at the beginning of DOTS-plus project did not occur yet.

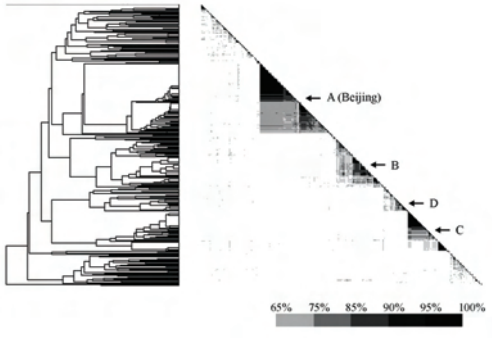
To gain insight in phylogenetic structure of M. tuberculosis population, the genetic lineages/sublineages and specific spoligotype signatures were defined in the collection of more than one thousand of well characterized strains by using internationally standardised spoligotyping procedure. Sixty five novel prototypes of spoligotypes were discovered. The most prevalent were strains that belonged to sublineages T1, Beijing, Haarlem 4, LAM 9, and Manu 2. As the result of spoligotyping the presence of large groups of highly virulent and genetically related strains have been confirmed by another independent method of genotyping. Overall, analysis of genotypes carried out by using both methods revealed that a limited number of specific M. tuberculosis strains cause the main portion of MDR TB cases in Lithuania.

The investigation of mutations conferring M. tuberculosis resistance to the second-line drugs used for the treatment of MDR TB was continued by additional search of 35 ofloxacin-resistant and 35 kanamycin-resistant strains. So far, the mutations in the gyrA and gyrB genes among 85 ofloxacin-resistant strains were determined by direct sequencing. Two thirds of them carried drug resistance determining mutations in the gyrA gene. A spectrum of these mutations observed among strains from Lithuania were consistent with distribution tendencies of mutations observed among isolates from different countries. Unfortunately, it was demonstrated that significant part of drug-resistant strains possessed resistance determining mutations in the gyrB gene. Therefore, the novel molecular techniques for fast prediction of M. tuberculosis resistance to ofloxacin should include fixation of the specific mutations in the both gyrA and gyrB genes obviously. Currently, the collection of kanamycin-resistant M. tuberculosis consists of 118 strains including 26 carrying cross resistance to amikacin and ten with a triple resistance to both aminoglycosides and capreomycin. However, only 15% of these strains had specific mutations conferring resistance to these drugs in the 5' region of rrs gene. None of capreomycin-resistant strains had mutation in tlyA gene as well. These results confirmed that the majority of M. tuberculosis clinical strains acquire resistance to reserve antituberculosis aminoglycosides and capreomycin by yet undiscovered molecular mechanisms.

This work was supported by the Lithuanian State Science and Studies Foundation (grants C-20/2006, C-01/2007 and T47/07).



Electron microphotograph of M.tuberculosis



Dendrogram and similarity matrix showing relatedness of M.tuberculosis strains circulating in Lithuania

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Grants

EU Framework 6th Programme
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NorFa network

Contracts

UAB Fermentas, Vilnius, Lithuania
Abcam Ltd, Cambridge, UK
Santa Cruz Biotechnology, USA
Customs Laboratory, Vilnius, Lithuania

Publications 2006-2007

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Patent

Zvirbliene A., Gedvilaite A., Ulrich R., Sasnauskas K. A mean to produce monoclonal antibodies; hybridoma cell lines generated by this mean, and recombinant chimeric virus-like particles with inserted foreign protein fragments as immunogens to generate hybridomas by this mean. Patent No. 5414 (Lithuanian Patent Office, 25.04.2007).

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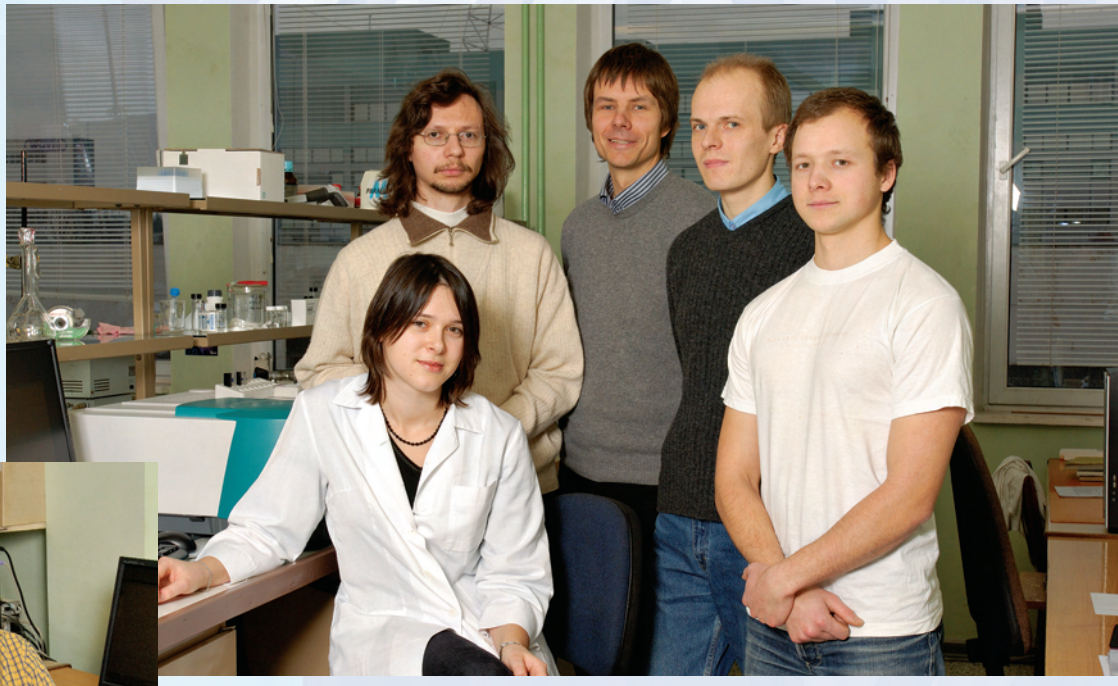
Laboratory of Biothermodynamics and Drug Design



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Founded in 2006 instead of the former Laboratory of Recombinant Proteins, the Laboratory of Biothermodynamics and Drug Design (LBDD) seeks to design, synthesize, and characterize novel chemical compounds with anticancer activity. Two classes of compounds were designed in 2006-2007 by structural biothermodynamics methods and experimentally tested by biophysical methods to be active inhibitors of enzymes that are anticancer targets. Two patent applications and several scientific publications were submitted. The advantage of the LBDD is the capability to design compounds in silico and experimentally test them by novel biophysical methods.

The laboratory's personnel is divided into five groups according to their activities:

The Group of Molecular and Cellular Biology is responsible for drug target protein production by cloning the genes of selected target proteins, their expression in E.coli, insect, or mammalian cells, and chromatografic purification of large quantities of active proteins sufficient for biothermodynamic measurements of binding with synthesized ligands. Several projects involve the design of protein domain constructs to alleviate insolubility problems. Protein production often involves their reconstitution and refolding from insoluble inclusion bodies, characterization of protein stability, and the measurement of enzymatic activity.

The Group of Biophysics is responsible for designing thermodynamic models and experimental measurements of chemically synthesized and natural ligand binding to target proteins by the following biophysical techniques: isothermal titration calorimetry (ITC) and protein melting temperature shift (thermal shift, TS, ThermoFluor). The group is equipped by isothermal titration calorimeters, differential scanning calorimeters, temperature-controlled fluorimeters and spectrophotometers. Furthermore, the group studies protein denaturation by high pressure using high pressure fluorimeter.

The Group of Molecular Modeling is responsible for designing thermodynamic models of chemically synthesized ligand binding to target proteins. Molecular modeling of candidate compounds often predicts novel compounds with improved binding capabilities. The group, together with several collaborating scientist is developing the software that estimates the energetics of ligand binding to a protein when only the crystal structure of free protein is available.

The Group of Organic Synthesis makes compounds that are designed to bind target proteins either by comparison with compounds of similar chemical structure or by computer simulation and molecular modeling. Special interest and capabilities of the group are in the field of synthesis of compounds with multiple conjugated aromatic heterocycles.

The Group of Industrial Biotechnology was established in 2007 with the start of the Lithuanian National Programme on Industrial Biotechnology (2007-2010). The group investigates technical applications and immobilization of enzymes for the production of biofuels and for starch transformations. Research on metabolic engineering and organic synthesis by fermentation are planned for the nearest future.

Research Projects

Carbonic anhydrases as anticancer targets

Carbonic anhydrases (CAs), a group of ubiquitously expressed zinc containing enzymes, are involved in numerous physiological and pathological processes, including gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity and the growth and virulence of various pathogens. In addition to the established role of CA inhibitors as diuretics and antiglaucoma drugs, it has recently emerged that CA inhibitors could have potential as novel anti-obesity, anticancer, and anti-infective drugs (Supuran, 2007).

CAs catalyse a simple reaction – the conversion of CO2 to the bicarbonate ion and protons. There are 14 CA isoenzymes in humans, three of them are inactive CA-like proteins that do not contain zinc and thus do not catalyse the CO2 hydration reaction. Expression of the 11 active CAs is variable in various tissues. The activity of CAs is quite variable too. A number of CA inhibitors, unsubstituted sulfonamides, have already been designed. However, most present inhibitors are insufficiently selective for target CA isozymes, such as hCAIX and hCAXII, anticancer targets.

Here at the LBDD we have designed a novel class of carbonic anhydrase inhibitors that exibit selectivity towards hCAIX over hCAI and hCAII. The inhibitors bind CAs with up to double digit nanomolar dissociation constants and significant selectivity towards specific isozymes.

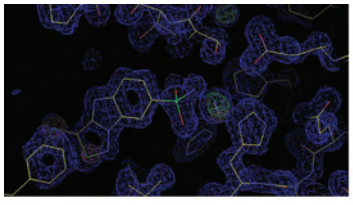


Fig. 1. *General formula of carbonic anhydrase inhibitors and the crystal structure of the inhibitor bound to the active site of hCAII (crystal structure was solved in collaboration with dr. Saulius Gražulis, Laboratory of Protein-DNA Interaction).*

Inhibition of Hsp90 chaperone

Heat shock protein 90 (Hsp90) is a molecular chaperone that is responsible for the correct folding of a large number of proteins. Client proteins of Hsp90 include many overexpressed oncogenes that are critical for the transformed phenotype observed in tumours.

We are interested in the mechanism of Hsp90 action and the thermodynamics of inhibitor binding. A novel group of inhibitors has been designed and synthesized that is similar to radicicol, a well known natural inhibitor of Hsp90.

A novel model based on the thermal shift assay was designed to determine the binding constants of tightly-binding ligands based on the Hsp90-radicicol binding. Upon binding radicicol, Hsp90 exhibits a double thermal transition.

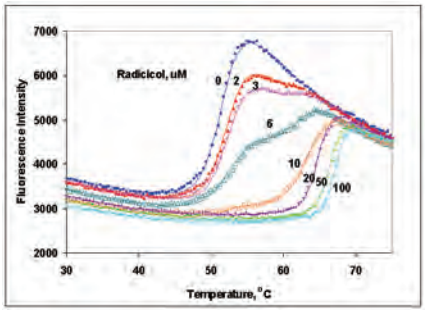


Fig. 2. *Hsp90-radicicol binding by the thermal shift assay*

Characterization of protein stability in the presence of various excipients

To determine the stability profiles of a recombinant protein, we use a miniaturized high-throughput thermal shift assay (also called ThermoFluor®) [Matulis D. et al. Biochemistry, 2005, 44, 5258-66]. The assay measures protein melting temperature by following the fluorescence of probes such as 1,8-anilino naphthalene sulfonate. The following picture shows the stability profile of hCAII in various buffers at various pHs.

The effect of a number of excipients such as osmolytes, organic solvents, amino acids, common metal cations and anions, lipids, and other chemicals and biochemicals on protein stability is tested in a single plate consuming several micrograms of recombinantly produced protein. The assay facilitates the determination of the optimal conditions for storage or other functional assays.

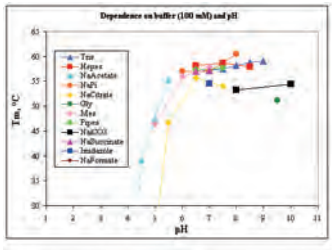


Fig. 3. *Stability profile of hCAII in different buffers at various pHs*

Determination of ligand binding constants by the thermal shift assay

The thermal shift assay allows us to measure ligand binding constants in high throughput while consuming micrograms of protein. Together with the isothermal titration calorimetry, we determine the full thermodynamic picture, including the Gibbs free energy,

enthalpy, entropy, and the heat capacity of selected binding reactions. The data is used for the design of more potently binding ligands.

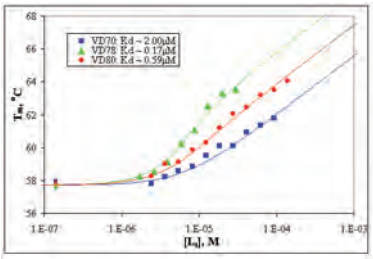


Fig. 4. *Determination of ligand binding constants to hCAXIII.*

Studies of Protein Denaturation by High Pressure

Pressure studies yield a variety of thermodynamic parameters that are unavailable by other methods, such as molar volume, expansion coefficient, and compressibility of the proteins in the native and denatured states. Ligand binding affects protein temperature and pressure stability. The pressure stability information yields novel methods of determining ligand binding equilibria.

Services

The LBDD is interested in the collaborations where our expertise in recombinant protein stability characterization and the determination of ligand-protein binding thermodynamics may be applicable. We can determine target protein thermal stability at hundreds of conditions in a single experiment by consuming microgram quantities of protein.

Collaboration

Prof. Seppo Parkkila, Institute of Medical Technology and School of Medicine, University of Tampere and Tampere University Hospital, Tampere, Finland
Prof. Claudiu Supuran, Universita degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Firenze, Italy
Prof. Maciej Zylicz, International Institute of Molecular and Cell Biology, Warsaw, Poland
Matthew J. Todd and Barry Springer, Johnson&Johnson, PRD, L.L.C., Philadelphia, USA, have donated ISS high pressure fluorimeter, Microcal MC2 isothermal titration calorimeter, Microcal MC2 differential scanning calorimeter, and protein purification chromatography system
Prof. Cathy Royer, Centre for Structural Biochemistry, Montpellier, France
Dr. Rolandas Meškys, Institute of Biochemistry, Vilnius, Lithuania
Prof. Sigitas Tumkevičius, Vilnius University, Faculty of Chemistry, Vilnius, Lithuania
Prof. Prutenis Janulis, Lithuanian Agricultural University, Kaunas, Lithuania

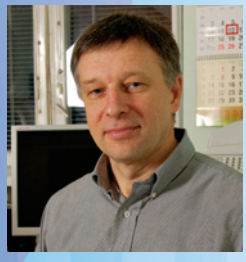
Grants

EC FP6 Marie Curie International Reintegration
Lithuanian State Science and Studies Foundation

Publications 2006-2007

1. Sereikaite J., **Jachno J.**, Santockyte R., Chmielevski P., Bumelis V. and **Dienys G.** Protein Scission by Metal Ion–Ascorbate System. Protein J., 2006 (25):369-378.
2. **Dudutienė V., Baranauskienė L., Matulis D.**. Benzimidazo[1,2-c][1,2,3]thiadiazole-7-sulfonamides as inhibitors of carbonic anhydrase. Bioorg. Med. Chem. Lett., 2007 (17): 3335-3338.
3. **Zaveckas M.**, Žvirblienė A., Žvirblis G., Chmieliauskaitė V., Bumelis V., Pesliakas H.. Effect of surface histidine mutations and their number on the partitioning and refolding of recombinant human granulocyte-colony stimulating factor (Cys17Ser) in aqueous two-phase systems containing chelated metal ions. J. Chromatogr. B, 2007 (852):409-419.
4. Romaškevič T., Budrienė S., Liubertienė A., Gerasimčik I., **Zubrienė A.** and **Dienys G.** Synthesis of chitosan-graft-poly(ethylene glycol) methyl ether methacrylate copolymer and its application for immobilization of maltogenase. Chemija, 2007 (18):33-38.
5. **Baranauskienė L., Matulienė J.**, and **Matulis D.** Determination of the thermodynamics of carbonic anhydrase acid-unfolding by titration calorimetry. J. Biochem. Biophys. Meth., in press.

Laboratory of Bioinformatics



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Rytis Dičiūnas, B.Sc.

Undergraduate students
Edita Bartaševičiūtė
Darius Kazlauskas
Mindaugas Laganeckas

PhD student
Jolita Višinskienė

Institute of Biotechnology
Centre of Excellence

In the post-genomics era bioinformatics is playing an increasingly important role in biological research. Breakthroughs in technologies have resulted in a flood of various types of biological data such as genome sequences for different organisms, data on gene expression, protein-protein interactions, etc. Bioinformatics is helping to make sense of all this vast biological data by providing tools that enable to perform large-scale analysis. In addition, bioinformaticians are utilizing available experimental data to improve various analytical and predictive methods that could help address specific biological problems.

The research carried out in our laboratory can be best described as Protein Structural Bioinformatics. There are two main research directions:

- Development of methods for protein modeling, assessment and analysis of protein structure as well as sequence search and comparison.
- Application of computational methods for structural/functional characterization of natural proteins and their complexes; design of novel proteins with desired properties. Our main focus are DNA-interacting proteins, in particular those functioning in DNA replication, repair and recombination.

There are a number of ongoing projects in the laboratory that cover diverse research topics. Here, several projects are described in more detail providing a flavor of our research.

Development of methods for protein homology detection

Re-searcher – a system for recurrent searching and reporting of new homologous sequences

Protein sequence searches are routinely employed to detect homologous proteins. However, at present, protein sequence databases are growing exponentially, necessitating frequent repetition of searches to find out whether new homologous sequences have been included. The analysis of results obtained during such repeated searches may also be tedious and time consuming. The task of manually keeping up with changes in databases becomes unbearable if one is interested in finding new homologs not for a single sequence, but for a few or few dozen sequences. To help cope with the periodic detection of new homologs we have developed Re-searcher, an open source sequence search and reporting system. Re-searcher uses PSI-BLAST, one of the most popular sequence search methods, as its search engine. The system can perform periodic searches against either locally installed or remote (NCBI, USA) databases. Re-searcher has a simple and intuitive interface, making the analysis of results even for a large number of queries a straightforward task. The software may be useful for both computational and experimental biologists and is freely available from our website (http://www.ibt.lt/bioinformatics/re-searcher/)

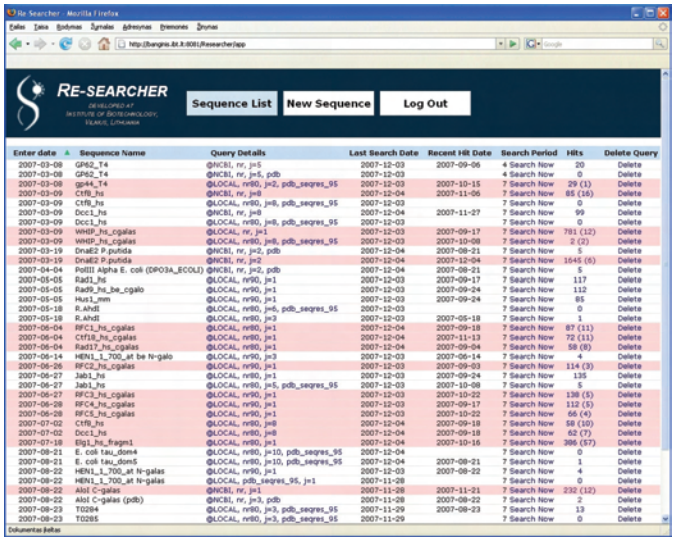


Figure 1. *A snapshot of the results window of the Re-searcher system*

Profile-based detection of remote evolutionary relationships between protein families

Related proteins tend to have similar three-dimensional shapes and molecular functions. Therefore, the detection of evolutionary relationship between protein families serves as a powerful technique for protein structural/functional annotation. This technique is used for both characterization of individual protein families and large scale annotation of protein coding genes in newly sequenced genomes. At present, protein sequence profile-based methods are among the most effective in detecting distant relationships between protein families. However, even those methods fail to identify many of the remote evolutionary links, underscoring the need for more sensitive approaches.

We accepted the challenging task of methods development, and decided to try improving homology detection by bringing in several new ideas. Our method, which at present is in the final stages of development, features a number of improvements. The improvements are introduced in all three major steps common to all profile-based methods: the construction of profile, the profile-profile comparison procedure, and the estimation of whether the compared profiles are related or not. Biologically more relevant treatment of insertions and deletions in profile alignments and rigorous statistical assessment of profile relatedness are among the most significant improvements. Although the development is not yet complete, our method is already capable of detecting very remote evolutionary relationships sometimes challenging even protein structure-based algorithms. The new method can successfully compete with other approaches that currently are considered to be top-of-the-line in remote homology detection. Therefore, we believe that it will contribute significantly to uncovering interesting novel links in the global proteome universe.

Application of computational biology methods to specific biological problems

Engineering of novel enzymes for biotechnology

One of the important practical tasks of computational biology is to drive the engineering of proteins with desired properties. Historically, our Institute has been always involved in the research of restriction enzymes that have become indispensable tools in DNA manipulation. One of the long standing goals is to understand how these enzymes recognize their DNA targets, and use that knowledge to produce novel enzymes with altered DNA specificity. The ability to produce restriction enzymes with desired DNA specificity would be important both for biomedical research and industry.
Working towards this goal we took part in a collaborative computational-experimental effort attempting to rationally design and then construct the world's first man-made type II restriction enzyme recognizing entirely novel DNA sequence. Representatives of type IIB restriction enzymes were chosen as the design material based on their discovered modular structural/functional architecture. In type IIB enzymes DNA cleavage and DNA recognition functions reside in separate regions of the polypeptide chain, allowing independent manipulation of these two functions. The advantage of using type IIB enzymes was also in that they use two independent structural domains for DNA recognition. Therefore, it was possible to design a novel enzyme with altered DNA specificity by employing the "Lego" approach. In this approach individual protein domains are replaced with those available in other naturally occurring proteins. The approach turned out to be very successful. Even the first in silico designed construct was functional, but after just a single amino acid substitution, suggested by the computational analysis, the properties of the new enzyme improved considerably. These results have demonstrated that computational methods may be very effective in the modification of existing proteins or construction of novel ones.

Computational prediction of protein structure and function

Computational methods are often used to predict three-dimensional structure and/or function for proteins as well as their individual domains. Next two projects illustrate the role of computational predictions in providing the basis for subsequent experimental studies.
In one of these projects, a computational technique called "fold recognition" was used to predict the structure of poorly characterized type II restriction enzyme BpuJI. In addition to identifying the evolutionary link of BpuJI with archaeal Holiday junction resolvases, the computational approach suggested its putative active site residues. These residues were subjected to experimental test, which confirmed their functional importance. Moreover, the detected relationship to Holiday junction resolvases allowed making a rational explanation of some peculiar nucleolytic properties of this enzyme.
Another project had to do with the characterization of a particular region of the catalytic subunit of bacterial DNA replicative polymerase (pol III). The catalytic subunit is a large multidomain protein, for which the three-dimensional structure until recently was not known. Moreover, it was not even clear whether it is related to other known polymerases or not. Using computational techniques, namely homology detection and protein structure modeling, we have found that one of the catalytic subunit domains features the OB-fold. Computational analysis strongly suggested that the do-

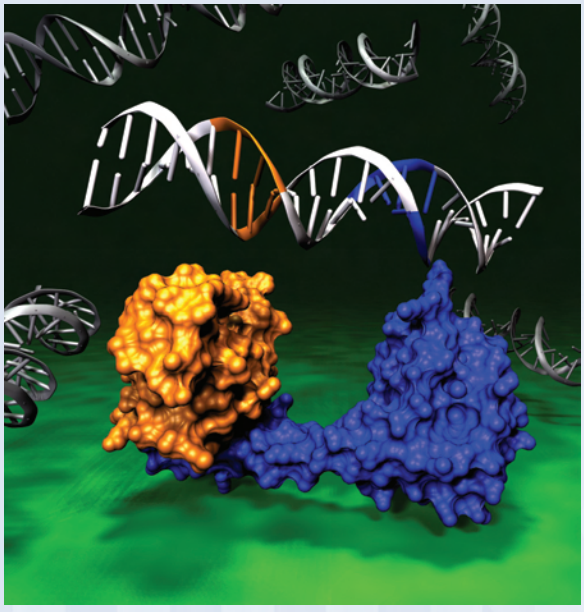


Figure 2. *A molecular model of the DNA recognition region of the new enzyme assembled in "Lego" style (orange and blue colors represent different pieces of the assembly)*

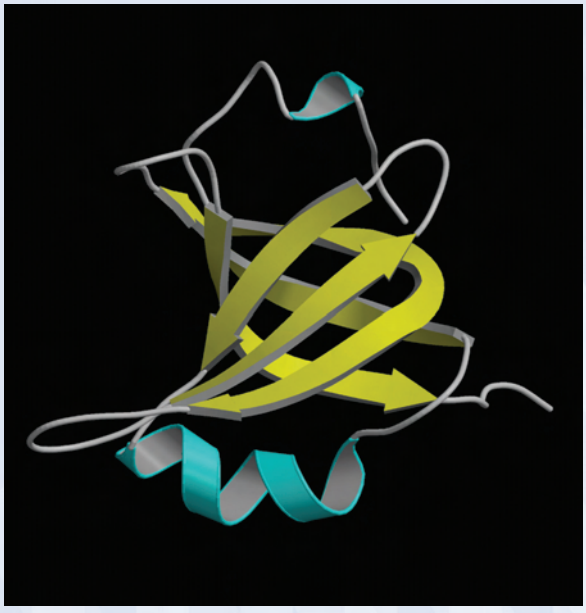
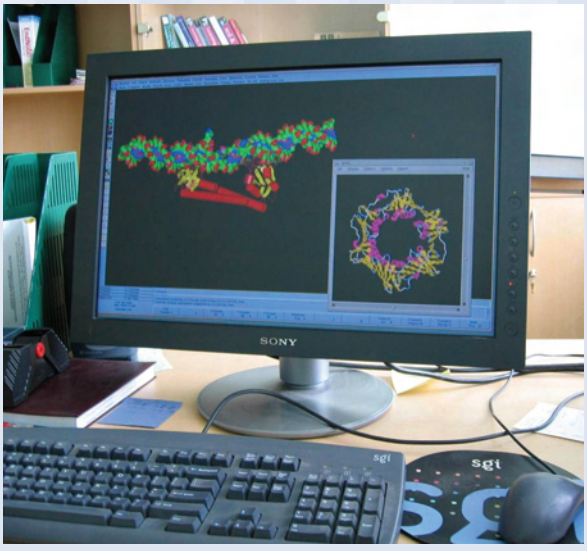


Figure 3. *Cartoon representation of a molecular model for the OB-fold domain of the E. coli replicative DNA polymerase.*

main may be able to bind single stranded DNA. Furthermore, it allowed us to identify putative DNA binding residues. Our collaborators from Northeastern University (Boston, USA) tested the prediction using a single-molecule DNA stretching approach in dual-beam optical tweezers experiments. The experiments confirmed the predicted ability of this region of catalytic subunit to bind single stranded DNA. Altogether these studies have shed light onto functional properties of individual regions of catalytic subunit thus advancing our understanding of DNA replication machinery in bacteria.



Some of the laboratory's computational resources: SGI computer graphics workstation and Hewlett-Packard Linux computer cluster (50 processors).

Collaboration

Dr. Penny Beuning, Northeastern University, Boston, MA, USA
Dr. Daniel Barsky, Lawrence Livermore National Laboratory, Livermore, CA, USA
Dr. Krzysztof Ginalski, Warsaw University, Warsaw, Poland

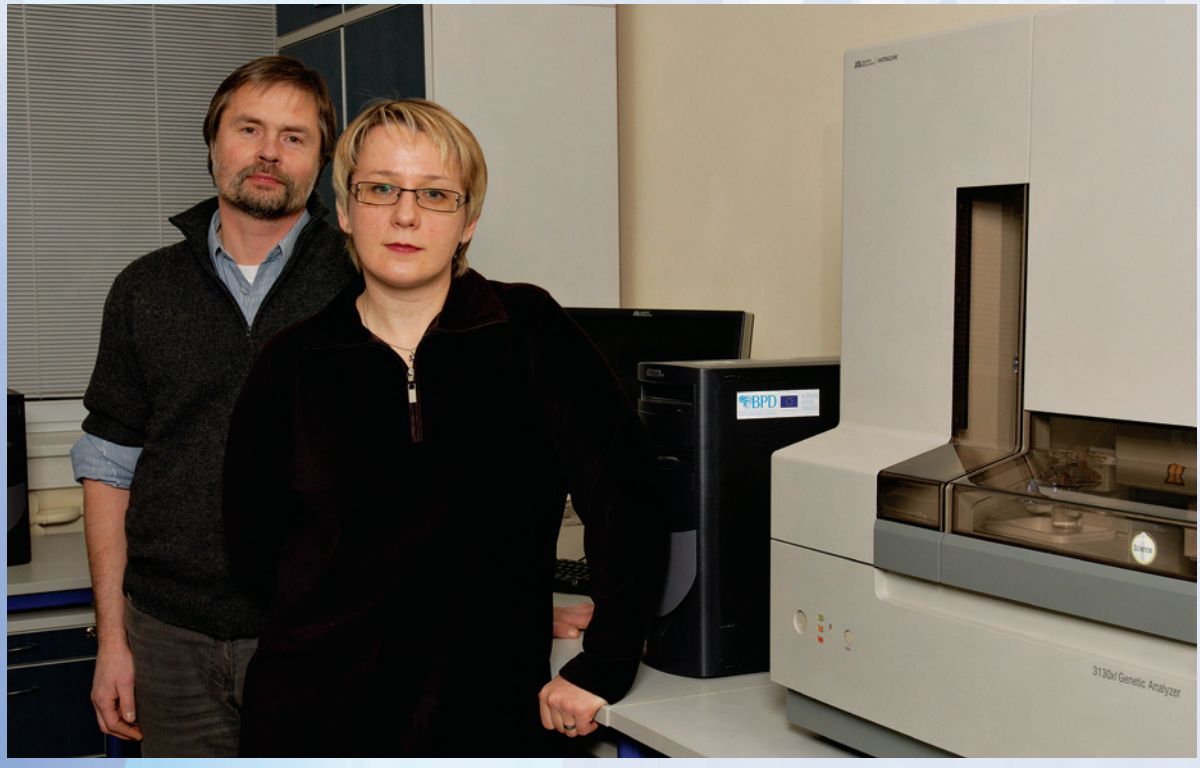
Grants

Howard Hughes Medical Institute
EC Framework 6 Programme
Lithuanian State Science and Study Foundation

Publications 2006-2007

1. Shi G., Chang D.Y., Cheng C.C., Guan X., **Venclovas Č.**, Lu A.L., Physical and functional interactions between MutY homolog (MYH) and checkpoint proteins Rad9-Rad1-Hus1. Biochem J., 2006 400 (Pt 1):53-62.
2. Rudokaitė-Margelevičienė D., Pranevičius H., **Margelevičius M.** Data Classification Using Dirichlet Mixtures. Information Technology and Control, 2006 (35):157-166.
3. **Timinskas A.**, Kučinskienė Z.A., Kučinskas V. Atherosclerosis: alterations in cell communication. Acta Medica Lituanica, 2007 (14):26-31.
4. Sukackaitė R., Lagunavičius A., Stankevičius K., Urbanke C., **Venclovas Č.** and Šikšnys, V. Restriction endonuclease BpuJI specific for the 5'-CCCGT sequence is related to the archaeal Holliday junction resolvase family. Nucleic Acids Res., 2007 (35):2377-2389.
5. Jurėnaitė-Urbanavičienė S., Šerkšnaitė J., Kriukienė E., Giedrienė J., **Venclovas Č.** and Lubys A. Generation of new DNA cleavage specificities of Type II restriction endonucleases by reassortment of target recognition domains. Proc. Natl. Acad. Sci. USA, 2007 (104):10358-10363.

DNA Sequencing Center



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Institute of Biotechnology
Centre of Excellence

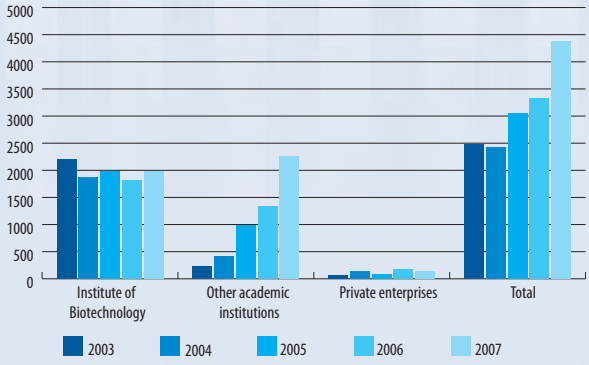
The DNA Sequencing Center (SC) was founded to help researchers, both at IBT as well as other institutions in Lithuania, process DNA samples in an efficient and economical manner. Located at the Institute of Biotechnology, the DNA Sequencing Center is successfully running since March 27 of 2003. The Center is equipped with the Applied Biosystems 3130xl Genetic Analyzer 16-capillary automated DNA sequencer that yields 700 to 1000 bases per template. SC performs cycle sequencing reactions using fluorescent dye terminators ABI Big Dye® Terminator v3.1 on any kind of DNA (plasmid, phage or PCR product) provided by the users. We also run the user's reactions. Usually, turnaround time takes 1-2 days after the receipt of samples. Sequencing of the larger samples may take longer.

Services provided by the DNA SC include:

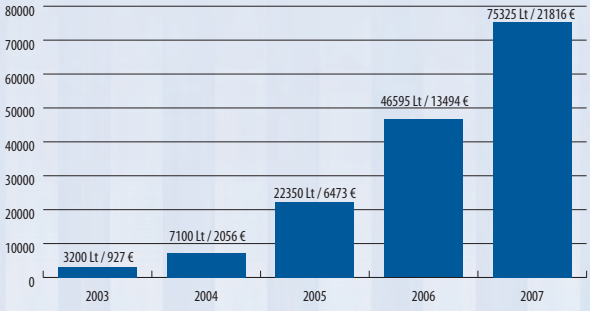
- Custom DNA Sequencing
- Sequencing and PCR troubleshooting and training workshops

We are committed to giving every user satisfactory sequence.

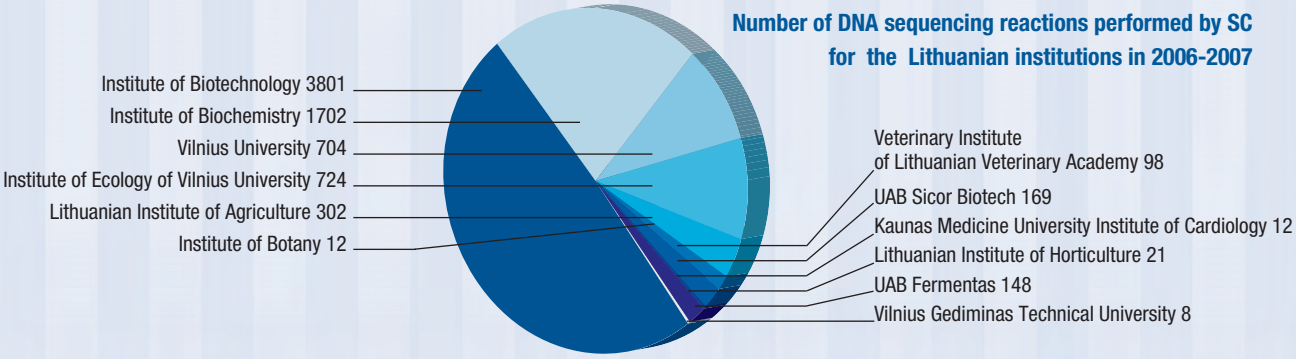
Number of DNA Sequencing reactions accomplished by SC in 2003-2007



Sequencing income for 2003-2007



Number of DNA sequencing reactions performed by SC for the Lithuanian institutions in 2006-2007



Addendum

European Union Grants

Framework 6 programme

Title	*Duration*
Meganucleases for Gene Replacement	2006-2008
A Multidisciplinary Approach to the Study of DNA Enzymes down to the Single Molecule Level	2005-2009
Targeting Newly Discovered Oxygen Sensing Cascades for Novel Cancer Treatments, Biology Equipment, Drugs	2004-2008
Inhibition of Cancer by Disrupting Interaction Between Polo-like Kinase 1 Polo-box Domain and Spindle Targets	2006-2008
Molecular Modeling-Based Characterization of Protein Complexes Involved in DNA Repair	2004-2006
Drug Design by Structural Thermodynamics	2004-2006
ScanBalt Competence Region – a Model Case to Enhance European Competitiveness in Life Sciences, Genomics and Biotechnology for Health on a Global Scale	2004-2006
Rational Design and Comparative Evaluation of Novel Genetic Vaccines	2004-2008

Framework 5 programme

Title	*Duration*
Enhanced Laboratory Surveillance of Measles	2002-2005
Support for the Integration of “Newly Associated States” in the European Research Area “Biotechnology Centre of Excellence of Lithuania	2003-2006

Structural funds

Title	*Duration*
Agricultural and Forest Biotechnology Research Network	2005-2006
Improving Quality of Human Resources in Agricultural Biotechnology and Forestry Investigations	2005-2006
Training of Postgraduates and Ph. D. Students in Agricultural and Forest Biotechnology	2005-2006
Strengthening of Experimental Basis for Interdisciplinary Research in Material Science, Biotechnology and Environmental Investigations	2005-2006
Improving Researchers Skills in Material Science, Biotechnology and Environmental Investigations	2006-2007
Introducing Research on Stem Cells and Cells of Higher Differentiation to Scientific Community	2005-2006
Building of Infrastructure for Proteomics Research	2007-2008
Improving Researchers Skills in Proteomics	2007-2008
Improving Quality of Human Resources in Research and Innovation	2006-2007
Developing Skills and Competence of Researchers and Experts in Genomics for Cardiology	2005-2006
Establishment of Post Doc Internship in Natural Sciences	2006-2008
Gaining Practical Skills in Biotechnology during Postgraduate Studies	2006-2008

International Grants

Howard Hughes Medical Institute

Title	*Duration*
Structural characterization of protein interactions in DNA replication, repair and recombination processes through molecular modeling,	2006-2010

Other International Grants

Title	*Duration*
International Research Training Group - Enzymes and Multienzyme Complexes Acting on Nucleic Acids	2006-2010
Wellcome Trust - Cross-talk between functional domains of Bfil restriction endonuclease	2005-2006
EuroTB - Molecular surveillance of multi-drug resistant tuberculosis in Europe	2003-2008

National Grants

Lithuanian State Science and Studies Foundation

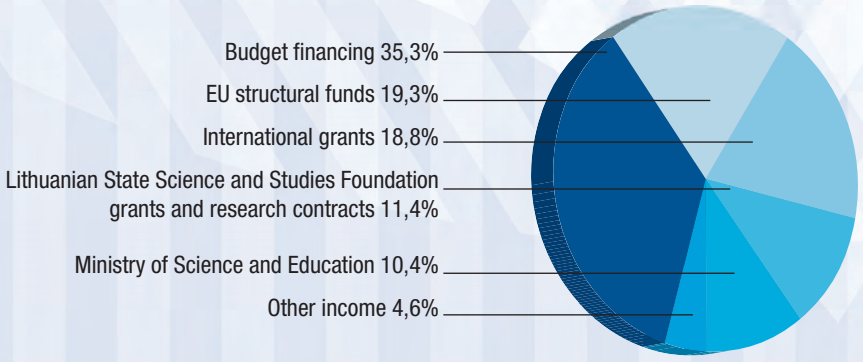
High-Technology Development

Title	*Duration*
New molecular tools for biotechnology	2003-2006
Enhanced surveillance of respiratory viruses	2003-2006
Generation of new monoclonal antibodies directed to desired epitopes using chimeric virus-like particles	2005-2006
New enzyme technologies for epigenome studies	2007-2009

Industrial Biotechnology

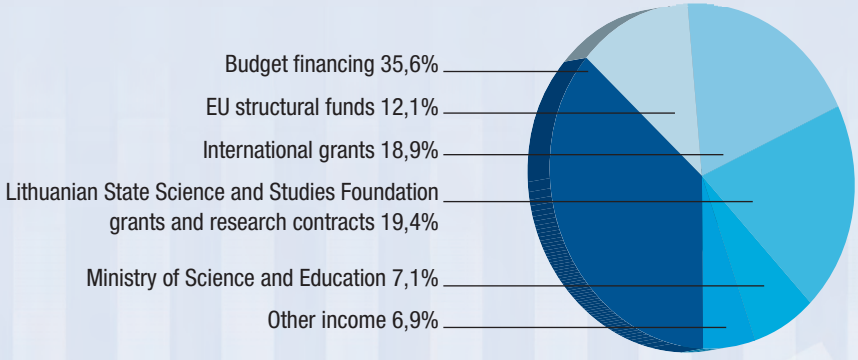
Title	*Duration*
Search for novel biofuel components and technological investigations to produce second generation biofuel	2007-2009
Design of technologies of recombinant proteins for prolonged therapy	2007-2009
Use of Biotechnological Methods For Carbonic Anhydrase Inhibitors Search	2007-2009
Engagement of metagenomic analysis of extremophile viruses from hot underground waters of Lithuania searching for the new enzymes	2007-2009
Detection Of Phytoplasmas And Viroids In Plants Valuable For Industrial Biotechnology And Their Removal	2007-2009

Financing sources, 2006



	Lt	*EUR*
Budget financing	3758800	1088624
EU structural funds	2049576	593598
International grants	1992955	577200
Lithuanian State Science and Studies Foundation grants and research contracts	1210203	350499
Ministry of Science and Education	1098195	318059
Other income	492971	142774
TOTAL	**10602700**	

Financing sources, 2007



	Lt	*EUR*
Budget financing	4240900	1228250
EU Structural funds	1444844	418456
International grants	2247968	651057
Lithuanian State Science and Studies Foundation grants and research contracts	2305801	667806
Ministry of Science and Education	844457	244572
Other income	823492	238500
TOTAL	**11907462**	

Doctoral thesis defended at the Institute of Biotechnology in 2006-2007

E.Kriukienė (on the left) and G.Lukinavičius defend Ph. D. thesis

2006

Slibinskas R. Synthesis of Mumps and Measles Virus Proteins in Yeast and Their Use in Diagnostics. Scientific supervisor: Prof. K. Sasnauskas.

Daujotytė D. DNA binding and active base flippinng by the Hha methyltransferase. Scientific consultant: Prof. S. Klimašauskas.

Pozdniakovaitė N. Human P14.5 Gene Characterization in Normal and Tumor cells. Scientific consultant: Dr. V. Popendikytė.

Zaremba M. Structure – stability – function correlations within the tetrameric restriction endonuclease Bse634I. Scientific consultant: Prof. V. Šikšnys.

Merkienė E. DNA Methyltransferase HhaI: Conformational Changes and Interactions with Substrates. Scientific consultant: Prof. S. Klimašauskas.

2007

Tamulaitienė G. Crystallographic and Functional Investigations of Type II Restriction Endonucleases Eco57I and SdaI. Scientific consultants: Prof. V. Šikšnys, Dr. S. Gražulis.

Jakubauskas A. Domain Organization Analysis of Type II Restriction Endonucleases. Scientific consultant: Prof. V. Šikšnys.

Lukinavičius G. Sequence-Specific Labeling of DNA via Methyltransferase-directed Transfer of Activated Groups (mTAG). Scientific consultant: Prof. S. Klimašauskas.

Kriukienė E. Restriction Endonuclease MnlI – a Member of the HNH family of nucleases. Scientific consultant: Dr .A. Lubys.

A new "born" doctor M. Zaremba with official opponents Prof. S.Klimašauskas (on the left) and Prof. A.Pingoud

Just "released" doctor G.Tamulaitienė with scientific consultants Prof. V.Šikšnys (on the left) and Dr. S.Gražulis

STRUCTURE AND MANAGEMENT OF THE INSTITUTE OF BIOTECHNOLOGY

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Printed by ***Mardosų spaustuvė***
Photographs by ***Arūnas Maniušis and the Institute of Biotechnology***

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