Institute of Biotechnology
Centre of Excellence

MoBiLi

SEVENTH FRAMEWORK PROGRAMME

35th anniversary

Report 2008 - 2010

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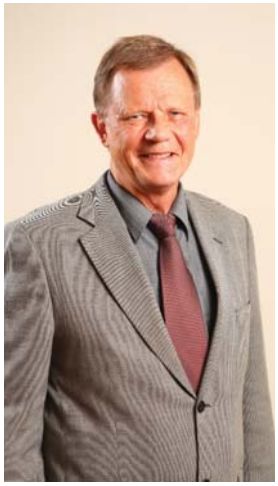


Director's note

This triennial report is dedicated to the 35 th anniversary of the Institute of Biotechnology. A completely new generation of scientists has evolved in thirty five years. The brief overview of the history of the Institute is presented on next pages. In my opinion, the most important happening in our history is the creation of Lithuanian modern biotechnology industry, which is competitive on the world market. We take exceptional pride of our internationally recognized spin-offs UAB Fermentas (currently ThermoFisher Scientific), UAB Sicor-Biotech (currently Teva), UAB Biocentras, UAB Biok, and the new UAB Profarma (2007) and UAB Nomads (2010).

We are extremely lucky that our scientists get prestigious and financially gratifying research grants, namely, National Institute of Health, The Max Plank Institute, Wellcome Trust, Howard Hughes Medical Institute, as well as the 5th, 6th and 7th FP, EEA , and the Lithuanian Science and Study Foundation (currently the Research Council of Lithuania). The substantial financial support from these outside sources significantly contribute to the budget of IBT, the allocation of the state budget comprises only 31% of the income. IBT draws the attention of the scientific community by publishing 20-25 scientific papers in the leading international journals each year.

Both the National Integrated Programme of Biotechnology and Biomedicine and the Programme of Industrial Biotechnology were initiated by the scientists of the Institute. These programmes provide opportunities to purchase new equipment and consumables, and help to integrate research with industry.

We constantly think about the development of IBT. Recently we have won the project of 7th Framework programme (FP7- REGPOT-2009-1) MoBiLi (1.600.000 Euro), directed to the integration of European research entities into the European research area. This project provides the Institute with a reasonable mechanism for turnover among researchers and enables the influx of new scientists, providing the feeling of well-earned stability to the best and brightest. According to this project, nine scientists from abroad will be invited for 2-3 years to work in the laboratories of IBT.

The 35th anniversary and the triennial report mark the end of independent status of the Institute of Biotechnology, since it was integrated into Vilnius University on October 1st, 2010. From this day its official name becomes Vilnius University Institute of Biotechnology (VU IBT) and a new period of the development of the Institute begins. We hope that a tight connection between IBT and the study processes will be very useful for both, the Institute and students of Vilnius University and VU IBT will meet challenges of the forthcoming changes and become an outstanding member of the knowledge based society.

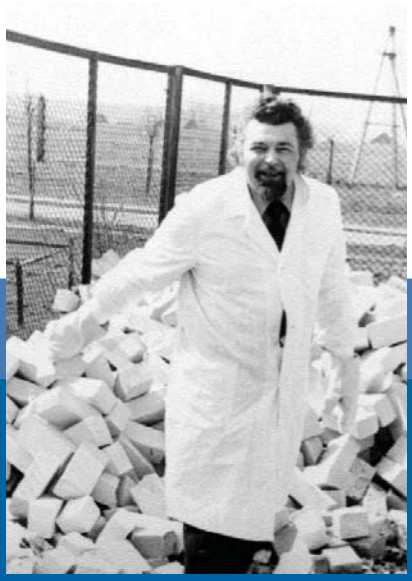
Prof. Kęstutis Sasnauskas

Institute of Biotechnology:
Just the Facts and Numbers

- Non-profit state budget Institute of Biotechnology (IBT) was established in 1992 after restructuring of the All Union Research Institute of Applied Enzimology.
- Located at V.A. Graičiūno 8, Vilnius.
- Total staff number is 145; research staff number is 82, it includes 48 researchers.
- The youngest Lithuanian research institute - average age – 37.
- Allocation of state budget comprises 31% of income; other 69% comes from outside sources (2009, grants and programmes).
- High level scientific research in step with applied research.
- Top level 20-25 scientific papers in peer reviewed high impact journals each year. Coming patent applications.
- Successful participation in EU FP (FP5, FP6, FP7) and other competitive international programs (HHMI, NIH, EEA). Only Vilnius University and Gediminas Technical University is ahead of IBT in revenue via competitive international programs.
- Selected as the Centre of Excellence in 2003 – EU FP5 tender - 600 000 Euros.
- The winner of the EU FP7 – Regional Research Potential: Coordination and Support action (FP7-REGPOT-2009—1) directed to the integration of European research entities into the European scientific research area - MoBiLi project – 1.600.000 Euros.
- Since 2000 after long term abroad 22 researchers have returned to IBT and were involved in the establishment of new laboratories at the institute.
- Involved in education of students at Vilnius University and Gediminas Technical University. Part of the institute lecturers are members of Committees on preparing Study Programs.
- 20—27 students accomplish Bachelor or Master thesis at IBT each year.
- Open to students for summer practice courses. Thirty eight, 15 and 14 students completed summer practice courses in 2007, 2008, 2009, respectively; Gene Engineering Laboratory for students was founded in 1999.
- Twenty students are currently involved in PhD studies at IBT; all in all, nine PhD theses were defended in 2008-2010. Main awards established by the Lithuanian Society of Young researchers and the Lithuanian Academy of Sciences were bestowed young researchers of IBT in 2007, 2008 and 2009.
- Famous Lithuanian Biotech spin-off companies emerged from the Institute (UAB Fermentas -1995, UAB Sicor - Biotech - 1995, UAB Biocentras - 1991, UAB Biok – 1991, UAB Profarma – 2007, UAB Nomads – 2010).
- Skilful personnel for the Lithuanian Biotech are trained at IBT; close connections with the Lithuanian Biotech industry are supported.
- National Biotechnology Program was initiated by IBT.
- Industrial Biotechnology Program was initiated by IBT.

An instant touch to the nineties

Photographs by Valdas Gerasimas




Palindromic Sequences
Restriction Endonucl

INSTITUTE OF BIOTECHNOLOGY:
Milestones 1975 – 2010

The history of the Institute of Biotechnology is very much the history of the modern biotechnology in Lithuania. Founded in 1975 as the all Union Research Institute of Applied Enzymology, having gone through a lot of restructuring, it has been carrying out the activities under the name of Institute of Biotechnology since 1995. A major difference exists between the Soviet (1975-1990) and post Soviet (1990-2010) periods of the Institute. In the first period, its activities depended upon the decision of the Central Board of Microbiological Industry of the Soviet Union Council of Ministers (Glavmikrobioprom) and were directed to the development of technologies for:

- production of highly purified enzymes for molecular biology, analytical and medical purposes;
- production of industrial (bulk) enzymes on a large scale and preparation of manuals for their application.

Bacterial strains producing well-known restriction enzymes and methyltransferases were obtained and the schemes for their purification were developed, thus providing home market with the tools for molecular biology. In parallel, a search for new restriction enzymes competitive on the international market was started on own initiative resulting in the discovery of 30% of restriction-modification enzymes currently known world-wide.

Besides the investigation of restriction enzymes the Institute was involved in the development of technologies for purification of enzymes for diagnostics and enzymatic analysis. Highly purified glucose oxidase was widely applied for the determination of glucose in biological liquids. Technologies for obtaining functionally pure phospholipases C and D, acetylkinase of a microbial origin were developed as well. In parallel, sorbents for enzyme purification were synthesized. Technologies for producing horseradish peroxidase, lactate-dehydrogenase, mitochondric malate-dehydrogenase, yeast glucose-6-phosphate-dehydrogenase and hexokinase, flavocytochrome b2, formate-dehydrogenase and other were developed. Usually, properties of these enzymes surpassed characteristics of similar enzymes produced by foreign companies.

Improvement and modification of methods for the measurement of enzymatic activities , development of new assay methods, as well as of methodologies for building substrate-detecting analytical equipment (biosensors) via the employment of immobilized enzymes, quality control of highly purified enzymes and studies of their physical-chemical properties were among the tasks of the Institute till 1990.

On the other hand, the Institute together with Vilnius Enterprise for Bulk Enzymes developed technologies for production of enzymes in large scale for use in industry and agriculture. For example, fungal enzymatic preparation Maltavamarin G10X for fur industry, bacterial glycosidase and lysozyme, multienzymatic compositions MEK-LP and MEK-GPL for poultry farming and cattle-rearing, multienzymatic composition MEK-1 for wine industry, acid-resistant Fermosorb and Polyferm for use in veterinary, and lysosubtilin G10X.

In 1984, the investigation of recombinant proteins for medical purposes and the development of their technologies were started at the Institute. Technologies for production of alfa2b-interferon, gama-interferon, beta-interferon, tumour necrosis factor and interleukin-2 were developed and the biological properties of these proteins were studied. Later on, the technology for production of recombinant human growth hormone was developed. Due to joint research with the Institutes of the Academy of Sciences of the Soviet Union, the tertiary structure of recombinant human growth hormone was determined for the first time.

In 1986, studies related to ecological biotechnology were initiated at the Institute. Investigations of Pseudomonas putida were carried out and the substance for decomposition of petroleum pollutants in the environment was developed under brand name Putidoil and tested closely with the scientists from the Siberian institutes.

In 1987, facilities for a large-scale production of highly purified enzymes were built. However, it became clear that the demand for such enzymes was lower than it had been anticipated, and the facilities were applied for the production of recombinant proteins for medical purposes. The technology for production of human recombinant alfa2b-interferon was developed and the interferon was registered in the USSR in early 1990 and in many other countries later on under the brand name Reaferon™ .

By 1990, a strong team of over 700 employees was built at the Institute. The basis for scientific research and international trade of restriction endonucleases and other enzymes involved in metabolism of nucleic acids was laid. Technologies for production of recombinant proteins were developed, new strains for production of enzymes were discovered, and many laboratory and pilot manuals for the manufacture of highly purified, industrial and immobilized enzymes were developed.

To keep up a high level of applied research, the fundamental investigations were also carried out in the Soviet years. Over two hundred and seventy scientific manuscripts in the all-union scientific journals ("Biochimija", "Molekuliarnaja biologija", "Bioorganičeskaja chimija", "Doklady AN SSR" etc.) and peer reviewed international journals ("Nucleic Acids Research", "Enzyme and Microbiological Technology", "Biotechnology and Bioengineering", "Applied Biochemistry and Biotechnology", "Chromatographia", "Biochemistry Journal", "Analytical Biochemistry", etc.), were published; 211 patents valid in the former USSR were granted.

In 1990, after re-establishment of independence of Lithuania and gaining the status of a state research institute, it became clear that a major part of developed products were not competitive on the international market, except for DNA restriction and modification enzymes, and several human recombinant proteins. Hence, restructuring was unavoidable for the survival of the Institute. **The reorganization was undertaken by separating basic and applied research with the establishment of the Molecular Biology Centre and the Centre of Genetic Engineering and Pharmaceuticals in 1993. In two years, these centers evolved into spin-offs, namely, Fermentas (presently ThermoFisher Scientific) and Biofa (presently Sicor Teva).** After two companies settled apart, the priorities of the Institute, whose name was shortened to the Institute of Biotechnology (IBT), have been focused and concentrated in the field of modern biology-biotechnology to:

- genetic and molecular studies of restriction-modification phenomenon;
- function of genes in yeast;
- research and development of recombinant biomedical proteins;

Genetic and molecular studies on restriction-modification (RM) phenomenon have been carried out at the institute since 1975. The phenomenon of taxon-specificity of restriction-modification has been discovered for the first time in the world. The new minor base N4-MT-cytosine, m4C, has also been discovered here. Over 30% of RM systems known in the world have been cloned at the Institute. Atypical RM systems and restriction enzymes have been discovered and attributed to the new type IVA. For the first time, it was demonstrated that restriction enzymes of class II, which recognise asymmetric sequences and split within their boundaries, can be formed from two non-identical subunits, and two separate MT-ases take part in methylation of the recognised sequence.

A vast contribution on investigations into the DNA restriction-modification phenomenon of the institute was acknowledged by the possibility to organize the international workshop on "Biological DNA Modification" in Vilnius in 1994 and include the Nobel Prize winner Dr. Richard J. Roberts (USA) into the list of invited speakers.

In depth structure-function studies of restriction endonucleases and methylstransferases were carried out to reveal the detailed mechanisms of specific DNA recognition and modification. Twelve crystal structures of restriction enzymes, comprising nearly one-third of restriction endonuclease structures known-to-date have been solved at the IBT (including joint collaborative projects). The knowledge gained during structural and functional studies of restriction enzymes have been used to engineer novel molecular tools for genome analysis and gene therapy.

The discovery and subsequent extensive studies of a phenomenon called DNA base flipping has greatly contributed to the understanding of salient mechanistic aspects of DNA methylation. A fluorescent and chemical method to determine flipped-out bases in DNA-protein complexes has been devised. A convenient technology of **mTAG** (methyltransferase-directed Transfer of Activated Groups) for targeted labelling of biomolecules has been designed thus providing a new way to specifically derivatize DNA sequence.

Protein Structural Bioinformatics has been added to the list of compentencies of IBT since 2005. Bioinformatics research at IBT includes the 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, and the design of novel proteins with desired properties. Application of computational methods is mainly focused on DNA-interacting proteins, in particular those functioning in DNA replication, repair and recombination. A number of computational methods developed as part of bioinformatics research are publicly available at http://www.ibt.lt/bioinformatics/software/ either as web servers or standalone software packages.

Despite the short history, **IBT bioinformaticians have already gained international recognition in the area of protein structure prediction. Template-based protein modeling results achieved by drs Č. Venclovas and M. Margelevičius during worldwide experiments on the Critical Assessment of Protein Structure Prediction (CASP) in 2005 and 2008 were ranked respectively as the second and the first. Often these CASP experiments are regarded as the Olympic games in protein structure prediction.**

Investigation of gene function in yeast was started in 1978. These investigations mainly involve the design of new expression systems of yeast genes and their application in obtaining human and virus proteins. Genes of yeast Saccharomyces cerevisiae (ADE1, ADE2, and CAD1), Candida maltosa (CYH-R, FDH1), Kluyveromyces marxianus (EPG1, TPI1) have been cloned and studied. Yeast strains producing human growth hormone, proinsulin, antigens of human hepatitis B virus surface and different chimeric proteins harbouring epitopes of antigens of human viruses have been constructed. Efficient synthesis of proteins encoded by human polyomavirus (JCV and BKV, Merkel carcinoma), paramyxoviruses (mumps, measles, henipaviruses, Menangle, Nipah, parainfluenzaviruses, RSV, metapneumovirus) , influenza A and B viruses, hantaviruses (Pumala, Dobrava and Hantaan serotypes), parvoviruses (Boca virus, PPV), circoviruses (porcine circovirus 1 and 2) in yeast have been obtained for the first time. It was demonstrated that these proteins form virus-like particles in yeast cells. Recombinant viral proteins were applied for the development of diagnostic kits of a new generation and the construction of new gene transfer systems in primary genetic therapy experiments. Serologic systems for testing viruses associated with respiratory infections were designed.

Research directed to the development of new diagnostic tools was performed. This includes antigenicity studies of recombinant viral proteins and the development of monoclonal antibodies. Hundreds of new hybridoma cell lines, producing highly-specific antibodies against viral and bacterial antigens, human cytokines, hormones, and cellular receptors, were developed and characterized. It was demonstrated that recombinant antigens and monoclonal antibodies represent safe and cost-effective reagents for serologic diagnosis of virus infections.

Studies on molecular epidemiology of drug resistant tuberculosis have been carried out by using internationally standardized spoligotyping procedure and restriction length polymorphism typing. These investigations provided new data on the prevalence of multidrug resistant Mycobacterium tuberculosis strains in Lithuania.

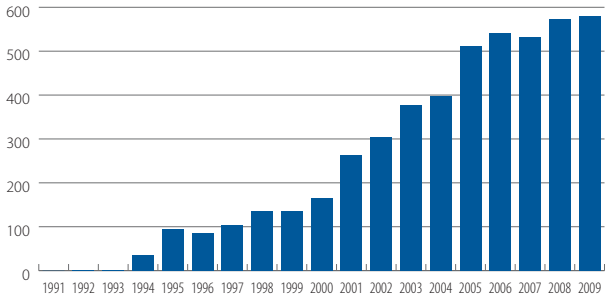
Studies on hypoxia-inducible factors and mRNA alternative splicing mechanisms in cancer cells have implications for better understanding of the processes involved in tumorigenesis and provide a novel source for the discovery of diagnostic or prognostic biomarkers as well as potential targets for therapeutic intervention.

Experimental structural studies at IBT have been expanded by structural biothermodynamics with the major goal of discovering promising compounds for anticancer drug development since 2005. Novel chemical compounds with anticancer activity were designed, synthesized and characterized. Several protein targets, such as the family of human carbonic anhydrases and the family of human chaperone proteins (especially Hsp90) were chosen as the primary targets of interest. Novel small-molecule compounds were designed in silico, synthesized by chemists, and their inhibition efficiency measured by novel biophysical techniques: isothermal titration calorimetry and the protein melting temperature shift assay.

Since 1990, IBT scientists published almost three hundred articles in international peer reviewed journals indexed by the Web of Science and cited world-wide. Six international patents were granted.

IBT citations in 1991 - 2009

ISI Web of Knowledge, Science Citation Report 2009



Articles, proceeding papers, review, editorial material found: 276; Sum of the Times Cited:4865;
h-index: 36. The citations indicated above concern the articles published mainly in the journals well known for scientific community: Cell, Nature, EMBO J, Journal of Biological Chemistry, Nucleic Acids Research, Journal of Molecular Biology, Journal of General Virology, Current Biology, Structure, Biochemistry, Proceedings of the National Academy of Sciences USA, etc..

Distinguished research at the Institute gained acknowledgement on the national level as well. **The Lithuanian Science Prize was awarded for a series of works to:**

prof. V. Butkus and prof. A. Janulaitis for "Studies of Bacterial Restriction-Modification System" in 1994;

prof. S. Klimašauskas and prof. V. Šikšnys for „Structural and Functional Studies of DNA Interacting Enzymes", in 2001;

dr. A. Ražanskienė, dr. A. Gedvilaitė and prof. K. Sasnauskas for "Synthesis of Virus Proteins in Yeast and their Application for Vaccines and Diagnostics", in 2003;

dr. Č. Venclovas for "Development and Application of Bioinformatics Methods for the studies of Protein Structure, Function and Evolution", in 2009.

The Institute has been collaborating with internationally renowned research centres from the USA, Germany, Sweden, Finland, England, Switzerland, Japan, France, Denmark, Canada, Poland, Latvia, and Estonia. Close co-operation has been maintained with the laboratories of the Nobel Prize winners Dr. Richard J. Roberts, Prof. Dr. Robert Huber, and Prof. R. M Zinkernagel. International co-operation has assisted in winning tenders and receiving the financial support for joint projects. The substantial financial support obtained from of the EU FP4, FP5,

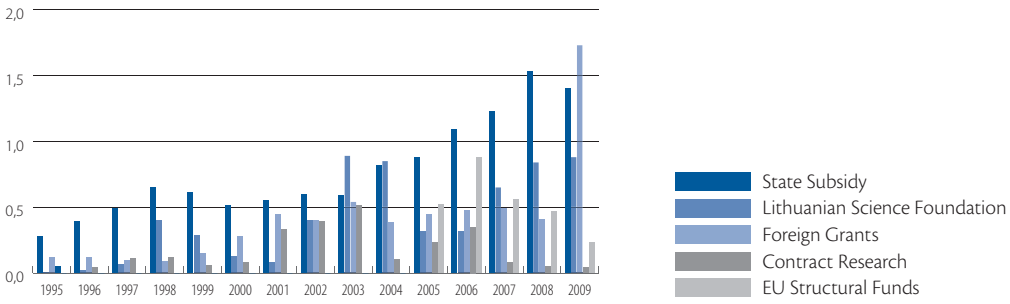
Subject areas covered:
Biochemistry and Molecular Biology (136)
Virology (35)
Biotechnology and applied Microbiology (31)
Biochemical Biophysics (19)
Cell Biology (23)

FP6 and FP7 programs, Inco Copernicus program, Volkswagen Stiftung, NATO, Wellcome Trust, Howard Hughes Medical Institute of Health, etc. contributed significantly to the budget of the Institute during 1995-2010.

The visibility of IBT has become more apparent and internationally recognised with the entering into the EU FP5 project "Support for the integration of "newly associated states" in the European research area "Biotechnology Centre of Excellence of Lithuania" in 2003. It allowed hosting of numerous principal researchers, graduates and postgraduates and helped to repatriate a number of Lithuanian skilful scientists from abroad. These scientists took destination home and brought novel ideas and innovative proposals to expand the list of competencies of IBT, and even establish two new laboratories (Lab. of Bioinformatics, and Lab. of Biothermodynamics and Drug Design). The Institute counts up 20 researchers, who have returned to IBT after a long-term stay abroad since 1995.

The IBT status of the Centre of Excellence added to a choice of the Institute for two major international events. In 2003, the USA National Academy of Sciences together with Howard Hughes Medical Institute granted IBT a right to host An Intensive Lecture/Laboratory Course on Molecular Interactions of Proteins and Nucleic Acids with prominent invited speakers and lecturers from Europe and the USA. It was attended by 20 postdocs from the Eastern European countries. Later, **in 2006, IBT hosted an intensive practical course on Directed Enzyme Evolution to researchers from the EU countries at early stages of their research careers: from Ph.D. students to junior postdocs.** Such courses speed up the pace of research by helping scientists learn about the latest techniques and findings from other laboratories and follow up collaboration.

Main Financing Sources, 1995 - 2009, Mln. EUR



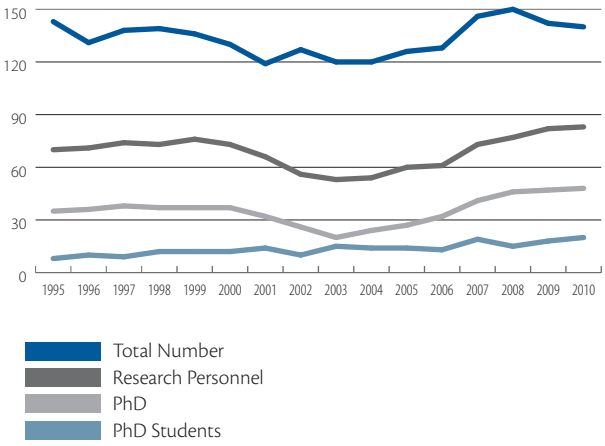
Ranking of IBT was among the highest according to the Questionnaire on the Selection of Centres of Excellence in Physical, Biomedical and Technological Sciences circulated by the Lithuanian Centre of Quality Assessment of Higher Education in 2007-2008.

The EU FP7 project "Strengthening and Sustaining the European Perspectives of Molecular Biotechnology in Lithuania" (MoBiLI) started in 2009 and occupies a very special place among earlier and future projects, because it allows concentrating on human capital building for R&D in the field of state-of-the art molecular biotechnology, networking of IBT with major centres of Excellence in the EU, upgrading and modernization of research infrastructure in line with emerging thematic priorities in the field and shows that success can only come with active internationally recognized group leaders, supported by teams of trained and enthusiastic fellows.

Besides contributing to molecular biotechnology research and development, scientists of IBT are involved in teaching students of Vilnius University, Gediminas Technical University, and Kaunas University of Technology. The up-to-date advanced level contemporary Gene Engineering Laboratory (total area of 158m2) for student training was founded at the Institute in 1999. The Lithuanian-Italian Bilateral Fund greatly contributed to the purchase of the necessary modern equipment. Young people take keen interest in Life Sciences. Since 2007, the IBT has been open to students for summer practice courses. Seventy students took an opportunity to get acquainted with research and facilities at the Institute during these courses in 2007-2010. Twenty to twenty seven students accomplish Bachelor or Master theses at IBT each year. Three to six graduates enrol in a four year Biochemistry or Chemical Engineering Ph.D programmes, have been offered by IBT together with Vilnius University or Gediminas Technical University since 1998. Main awards established by the Lithuanian Society of Young researchers and the Lithuanian Academy of Sciences for the best Ph.D thesis were bestowed to the young researchers of IBT, namely, dr. M. Zaremba (2006), dr. G. Tamulaitienė, dr. G. Lukinavičius (2007), and dr. G. Tamulaitis (2008).

Hundred and forty five people were employed at IBT at the end of 2010. These numbers comprised 82 persons who engaged in research and were thirty seven year old on average. **Despite of an outflow of highly-qualified and skilful researchers from IBT to its spin-offs UAB Fermentas (presently ThermoFisher Scientific) and UAB Biofa (presently Sicor Biotech), the number of scientists at IBT has increased since 2004 and reached 48 at the end of 2010.**

Personnel Number Change at IBT through 1995 - 2010



IBT has been active in the field of molecular biotechnology over the past 35 years. During this time, the institute became a recognized national leader in Life Sciences and in the whole Central and Eastern European region. Encouraged by results and growing recognition, IBT continues to strive for excellence by consolidating and focusing its competitive strength and core competencies in order to stimulate the development of high value strategic opportunities linked with the newly adopted Law on Study and Science and restructuring of RTD sector in Lithuania within the Concept of Integrated Science, Studies and Business Centres (Valleys) and compelling to meet new challenges after the integration into Vilnius University on the first of October 2010.

Directors of the Institute 1975-2010

Antanas Skaistutis Glemža, 1975 03 01 – 1978 01 16; 1988 12 28 – 1989 03 05 (acting director)

Donatas Kazlauskas, 1978 01 17 – 1987 05 11

Henrikas Dūdėnas, 1987 05 12 – 1988 12 26

Arvydas Eugenijus Janulaitis, 1989 03 06 – 1992 02 13

Vladas Algirdas Bumelis, 1992 02 14 – 1996 07 15

Algimantas Antanas Pauliukonis, 1996-07-16 –2007 06 26

Kęstutis Sasnauskas, 2007 06 27 -

Grants 1995-2010

FRAMEWORK 7 PROGRAMME	
Strengthening and Sustaining the European Perspectives of Molecular Biotechnology in Lithuania (MoBiLi)	2009-2013
Metastatic tumours facilitated by hypoxic tumour micro-environments (METOXIA)	
Development of novel antiviral drugs against Influenza (FLUCURE)	2010-2014
Pan-european network for the study and clinical management of drug resistant tuberculosis (TB PAN-NET)	2008-2013
Small molecule inhibitors of the trimeric influenza virus polymerase complex (FLUINHIBIT)	2008-2010
FRAMEWORK 6 PROGRAMME	
Meganucleases for gene replacement	2006-2008
Inhibition of cancer by disrupting interaction between polo-like kinase 1 polo-box domain and spindle targets	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
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	
Support for the integration of newly associated states in the European research area "Biotechnology Centre of Excellence of Lithuania"	2003-2006
Development of highly special enzymes for genome manipulation	2003-2004
Enhanced Laboratory Surveillance of Measles	2002-2005
Combined immune and gene therapy for chronic hepatitis	2001-2003
Comprehensive risk analysis of dioxins: development of methodology to assess genetic susceptibility to developmental disturbances and cancer	2000-2003
Bivalent hantavirus vaccine for Europe: Different approaches and evaluation in animal models	1999-2003
FRAMEWORK 4 PROGRAMME	
Molecular monitoring and pathological role of HCV, HGV and altered HBV genomes in the Baltic countries	1998-2001
Detection, identification and typing of the Mycobacterium tuberculosis in the Baltic countries	1998-2003
Recombinant viral particulate proteins as tools for new vaccines and diagnostics	1994-1997
HOWARD HUGHES MEDICAL INSTITUTE	
Structural characterization of protein interactions in DNA replication, repair and recombination processes through molecular modeling	2006-2010
Towards engineering of restriction enzymes	2003-2004
Bioinformatics-guided engineering of DNA methyltransferases	2003-2004
Sequence recognition and base flipping by DNA methyltransferases: Structural studies and redesign for novel functions	2001-2005
Principles of restriction enzymes specificity	2001-2005
Combination of improved methods with expert knowledge to derive models of protein structure at low sequence homology	2001-2005
Mechanisms of specific protein-DNA recognition and modification	1996-2000
NATIONAL INSTITUTE OF HEALTH	
Methylome profiling via DNA Methyltransferase directed labeling	2008-2010
Approaches for genomic mapping of 5-hydroxymethylcytosine a novel epigenetic mark in mammalian DNA	2010-2012
WELLCOME TRUST	
Cross-talk between functional domains of BfiI restriction endonuclease	2005-2006
Restriction enzymes with novel restriction mechanisms	2001-2003
Calorimetric studies of DNA- restriction enzyme interactions	1999-2001
INTERNATIONAL BUREAU OF THE BMBF	
New cofactors for methyltransferases	2004-2005
Human tumour vaccines based on chimeric virus-like particles	2002-2005
Bivalent Hantavirus vaccine for Europe based on recombinant proteins	2001-2003

VOLKSWAGEN STIFTUNG

Rational design and molecular evolution of DNA methyltransferases for new sequence-specific chemical modifications of DNA	2002-2004
Catalytic loop movements in DNA methyltransferases: fluorescence studies of intrinsically and extrinscally labeled mutants	1999-2001
Studies on the base-flipping mechanisms of DNA methyltransferases using fluorescent oligonucleorides	1996-1998

NATO

Natural resources for industry: Investigations of protein refolding factors and their implementation into biochemical process	1999-2002
13C-NMR characterization of intermediates on the base-flipping pathway of HhaI methyltransferase	1999-2002
Structure and function of restriction enzymes	1996-1998

EUROPEAN ECONOMIC AREA

Anticancer drug design by structural biothermodynamics	2008-2010

OTHER INTERNATIONAL GRANTS

The Royal Society, the European Science Exchange Programme: Correlating structure and spectroscopy: 2-aminopurine fluorescence from protein-DNA crystals	2002-2004
Nordic Country Cancer Society: Regulation of hypoxia induced factor activity via alternative pre-IRNA splicing	2002-2003
Swedish Institute for Infectiuos Diseases Control:Genotyping of Mycobacterium tuberculosis drug-resistant isolates from Lithuania	2001-2002
The Royal Swedish Academy of Sciences: mechanisms of expression and alternative splicing of a novel regulator of hypoxia signaling	1999-2000
Humboldt-Universität zu Berlin: Construction of new antigens on the basis of virus like particles	1998-2000
Max-Planck Institut für Biophysik: Investigation of the structure and function of peptide receptors and production of specific antibodies	1997-1998

STRUCTURAL FUNDS

Building of infrastructure for proteomics research	2007-2008
Improving skills of researchers in proteomics	2007-2008
Establishment of post doc internship in natural sciences	2006-2008
Gaining practical skills in biotechnology during postgraduate studies	2006-2008
Improving skills of researchers in material science, biotechnology and environmental investigations	2006-2007
Improving quality of human resources in research and innovation	2006-2007
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
Developing skills and competence of researchers and experts in genomics for cardiology	2005-2006
Introducing to scientific community investigations on stem cells and cells of higher differentiation	2005-2006

NATIONAL GRANTS

High-Technology Development Programme

Development of new tools for improved laboratory diagnosis of human papillomavirus (HPV) infection and HPV-related cancer	2009-2010
Development of Yeast expression system by using proteomic approach and gene engineering	2008-2010
New enzymes and technologies for epigenome analysis	2007-2009
Structural and functional studies of T4 phage replisome	2007-2009
Generation of new monoclonal antibodies directed to desired epitopes using chimeric virus-like particles	2005-2006
New molecular tools for biotechnology	2003-2006
Enhanced surveillance of respiratory viruses	2003-2006

Industrial Biotechnology Programme

Development of diagnostic tools for Merkel cell polyoma virus	2009-2010
Development of anti-cytolysin monoclonal antibodies designed to neutralize the toxic cytolysins of the pathogenic bacteria	2008-2010
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

Doctoral thesis, habilitation procedure 1997-2010

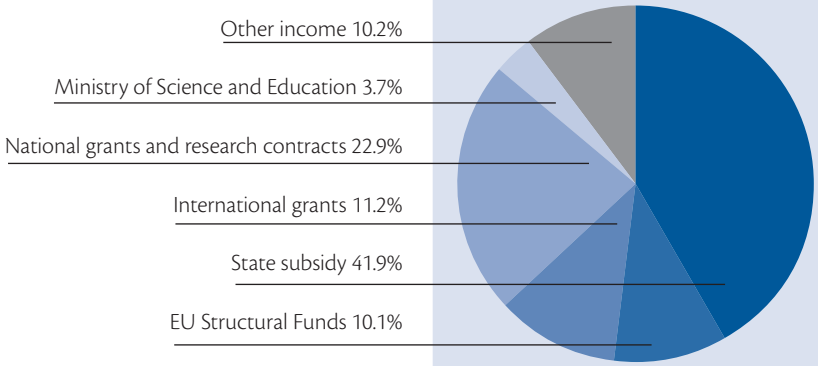
	Name	Title	Supervisor
1997	A. Lubys	Cloning and analysis of genes encoding type II restriction modification enzymes Bsp6I, Cfr9I ir HphI	Prof. A. Janulaitis
1999	A. Lagunavičius	Structural and functional relation of restriction endonuclease MunI	Dr. V. Šikšnys
2000	R. Skirgaila	Structural and functional relation of restriction enzyme Cfr10I	Dr. V. Šikšnys
	K. Stankevičius	Characterization of atypical type II restriction-modification enzymes Bpu10I and Eco47I-Eco47II	Prof. A. Janulaitis
	A. Timinskas	Comparative analysis of amino acid sequences of DNA methyltransferases	Prof. A. Janulaitis
	G. Vilkaitis	Functional and kinetic analysis of HhaI DNA methyltransferase ant its Thr-250 mutants	Prof. dr. S. Klimašauskas
2001	S. Serva	DNA base flipping by cytosine methyltransferase HhaI.	Prof. dr. S. Klimašauskas
2002	D. Bartkevičiūtė	Construction of systems for heterologous protein secretion in yeast	Prof. K. Sasnauskas
	J. Vitkutė	Screening and characterization of type II restriction enzymes	Prof. A. Janulaitis
2004	E. Mištinienė	Structure and properties of tumor associated antigen UK114 and its homologue protein p14.5	Prof. G. Dienys, Dr. V. Naktinis
	R. Rimšelienė	Construction of restriction endonuclease Eco57I mutants with altered sequence specificity	Prof. A. Janulaitis
	G. Sasnauskas	Novel subtype of type IIs restriction enzymes	Dr. V. Šikšnys
2005	M. Zaveckas	Partitioning and refolding of recombinant human granulocyte-colony stimulating factor in aqueous two-phase systems containing chelated metal ions	Dr. D. Matulis Dr. H. Pesliakas
2006	R. Slibinskas	Synthesis of mumps and measles virus proteins in yeast and their use in diagnostics	Prof. K. Sasnauskas
	D. Daujotytė	DNA binding and active base flippinng by the Hha methyltransferase	Prof. dr. S. Klimašauskas
	N. Pozdniakovaitė	Human P14.5 gene characterization in normal and tumor cells	Dr. V. Popendikytė
	M. Zaremba	Structure – stability – function correlations within the tetrameric restriction endonuclease Bse634I	Prof. V. Šikšnys
	E. Merkienė	DNA methyltransferase HhaI: conformational changes and interactions with substrates	Prof. dr. S. Klimašauskas
2007	G. Tamulaitienė	Crystallographic and functional investigations of type ii restriction endonucleases Eco57I and SdaI	Prof. dr. V. Šikšnys Dr. S. Gražulis
	A. Jakubauskas	Domain organization analysis of type II restriction endonucleases	Prof. dr. V. Šikšnys
	G. Lukinavičius	Sequence-Specific Labeling of DNA via methyltransferase-directed transfer of activated groups (MTAG)	Prof. dr. S. Klimašauskas
	E. Kriukienė	Restriction endonuclease MnlI – a member of the HNH family of nucleases	Dr. A. Lubys
2008	R. Petraitytė	Synthesis of viral proteins in yeast and their application for diagnostics	Prof. K. Sasnauskas, Dr. A. Ražanskienė
	A. Bulavaitė	Synthesis of chimeric polyomavirus MPyV, SV40 and hepatitis B virus surface proteins in yeast and the study of their properties	Prof. K. Sasnauskas
	L. Antoniukas	Large-scale production and purification of hantavirus nucleocapsid protein in yeast Saccharomyces cerevisiae for diagnostics and vaccine applications	Prof. K. Sasnauskas Prof. U. Reichl Habil. dr. R. Ulrich
	S. Jurėnaitė -Urbanavičienė	Enginnering of bifunctional restriction endonucleases with novel specifities	Dr. A. Lubys Dr. Č. Venclovas
	R. Šapranauskas	Investigation of type IIS restriction endonuclease BfiI domain organization by using a new random gene dissection approach	Dr. A. Lubys
	G. Tamulaitis	Structural and functional studies of the EcI18kI and EcoRII restriction enzymes specific for interrupted palindromic sites	Prof. dr. V. Šikšnys
2009	R. Sukackaitė	Structural and functional studies of the restriction endonuclease BpuJI	Prof. dr. V. Šikšnys Dr. S. Gražulis
2010	R. Ražanskas	Interaction of hepatitis B virus core protein and its mutant forms with human liver proteins	Prof. K. Sasnauskas

Habilitation procedure passed in Vilnius University

2005	V. Šikšnys	Structure and function of restriction endonucleases recognizing related sequences
2008	A. Gedvilaitė	Gene expression in yeast: molecular exploration and application
	A. Žvirblienė	Development and application of monoclonal antibodies

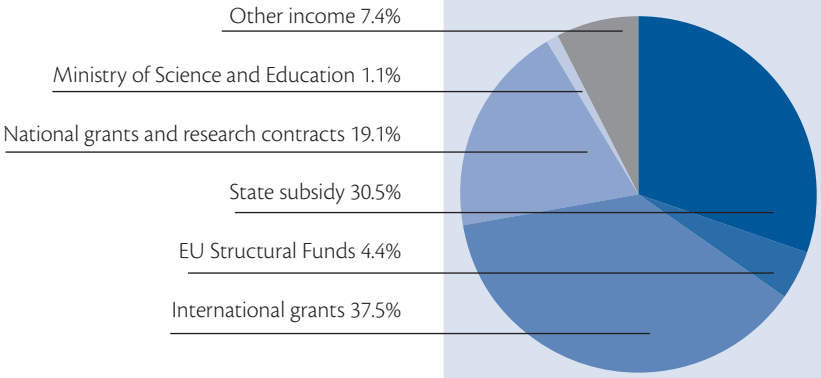
Financing sources 2008-2009

Financing sources, 2008



	Lt	EUR
State subsidy	5284200	1530410
EU Structural Funds	1273146	368729
International grants	1414316	409614
National grants and research contracts	2891116	837325
Ministry of Science and Education	463884	134350
Other income	1280592	370885
	12607254	3651313

Financing sources, 2009



	Lt	EUR
State subsidy	4845000	1403209
EU Structural Funds	688544	199416
International grants	5957354	1725369
National grants and research contracts	3034749	878924
Ministry of Science and Education	180905	52394
Other income	1176371	340701
	15882923	4600013

Strengthening and Sustaining the European perspectives of Molecular Biotechnology in Lithuania (MoBiLi)

MoBiLi is funded by the European Union, Research Potential Call FP7-REGPOT-2009-1

Mission of the MoBiLi: MoBiLi is a support action to strengthen the research capacities and to mobilize human resources in molecular biotechnology at the Institute of Biotechnology (IBT) Vilnius, Lithuania. The MoBiLi, dedicated to the strengthening and sustaining the European perspectives of Molecular Biotechnology in Lithuania, has been selected for funding by the EU FP7 Capacities programme. The latter coordination and support action (call FP7-REGPOT-2009-1) was very competitive: 312 projects were received by the Commission and only 16 were selected for funding (MoBiLi ranked 7-th).

Purpose of the project is to build up scientific excellence and human potential of IBT thereby transforming it into an excellence centre in molecular biotechnology and a significant player in the European Research Area.

The major objectives:

Human capital building for research and technological development (RTD) in the field of state-of-the-art molecular biotechnology

Networking of IBT with major centres of excellence in the EU via joint research and mobility of researchers

Upgrading and modernisation of research infrastructure in line with emerging thematic priorities in the field

The objectives of the project will be fulfilled by 7 Work Packages via collaboration with the **project core partners:**

The European Molecular Biology Laboratory (EMBL)

Karolinska Institutet, Stockholm (KI)

Justus Liebig University Giessen (JLU)

University of Edinburgh (UE)

The Swiss Institute of Bioinformatics (SIB)

Scientific priority areas of collaboration with the core partners cover topics like ***protein structure, interactions and cellular networks*** (JLU, EMBL, SIB, UE) and ***cellular imaging and high-throughput approaches to study human diseases*** (EMBL, KI, SIB, UE).

Project progress (December 2009 - September 2010)

Exchange of Know-How And Experience: During the period of 10 months, 6 scientists came to the IBT to do collaborative research and eight researchers from the IBT visited foreign partners. ;

Recruitment of Incoming Experienced Researchers: A series of job advertisements were placed in scientific journals and communicated through personal connections for the recruitment of incoming experienced researchers.

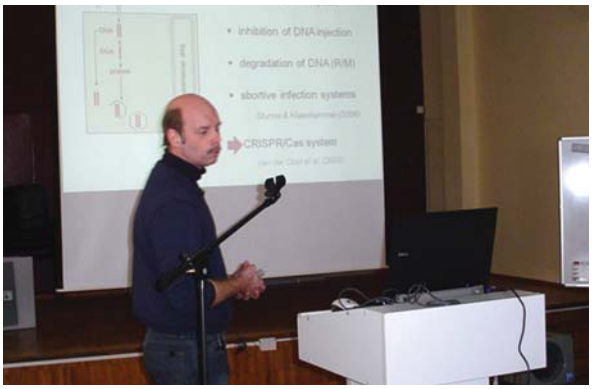
Nine scientists were selected from about 20 invited seminar interviews. Three of them, Group Leaders, will establish new research groups in the fields of Protein structure, interactions and cellular networks, High throughput approaches to study human disease, and Molecular, cellular biology, or biophysics. Remaining six scientists are young postdoctoral researches having gained significant experience abroad.

Acquisition, Development, Maintenance or Upgrading of Research Equipment: The MoBiLi project is aimed to create a stimulating, multidisciplinary environment promoting research of excellence in biomedicine at the interface between structural biology, chemistry and biology. Therefore IBT had purchased the following equipment: ***Universal X-Ray Difractometer, HPLC-MS System, Cell sorting system for high performance analytical and preparative flow cytometry and High performance computing (HPLC) Linux cluster.***

International Seminars & Workshops: Furthermore, MoBiLi aims to increase the international visibility of IBT, dissemination of scientific information obtained at IBT and exchange of know how with potential collaboration partners. 5 experienced researchers have already visited IBT and gave their presentations.

Nine IBT researchers had attended international conferences and workshops on structural and computational biology and biomedicine.

Dissemination and Promotional Activities: Dissemination activities will facilitate dissemination and transfer of knowledge at regional, national and international level involving both the own research/PR staff and invited specialists from other countries and will increase the in-



ternational knowledge/ experience exchange capacity and reputation of IBT. They will not only provide general information about MoBiLi and IBT as a whole, but will bring MoBiLi and IBT to an eyelevel position for future collaboration in research, e.g. EU FP7 as well as regional and national programs.

External Evaluation: To check and control the achieved research quality and scientific excellence at the project's end, an independent evaluation will be implemented. External evaluation facility is foreseen to take place after the end of the implementation in order to evaluate the applicant's overall research quality and capability. Four experts appointed by the Commission will visit the institution to discuss with the researchers and the research management in order to evaluate the capacity of the applicant.

Project Management: To ensure successful implementation and professional administration, vigorous project management is necessary. A project's kick-off meeting took place on March 26, 2010. First meeting of the project's Advisory Board members was held on the same day. The ***Advisory Board*** members are ***Prof. A. Tramontano*** (University of Rome "La Sapienza", Italy), ***Prof. A. Pingoud,*** (Justus-Liebig-Universität, Germany), ***Prof. L. Poellinger,*** (Karolinska Institutet, Sweden), ***Prof. S. Halford,*** (University of Bristol, U.K.), ***Prof. B. Samuelsson,*** (University of Gothenburg, Sweden), ***Prof. H. Grosjean,*** (University of Paris-South, France), ***Prof. E. Butkus,*** (Research Council of Lithuania), ***Mr. A. Markauskas,*** (Fermentas, CEO, Lithuania), ***Dr. A. Žalys,*** (Ministry of Education and Science, Lithuania), ***Prof. G. Dienys,*** (Lithuanian Biotechnology Association). The ***Management Board*** members are ***Mr. Leonas Pašakarnis,*** (Deputy Director of Institute of Biotechnology Vilnius), ***Prof. Saulius Klimašauskas,*** (Head of Laboratory of Biological DNA modification), ***Dr. Daumantas Matulis,*** (Head of Laboratory of Biothermodynamics and Drug Design), ***Dr. Gintautas Žvirblis,*** (Head of Laboratory of Eukaryote Genetic Engineering), ***Dr. Aurelija Žvirblienė,*** (Head of Laboratory of Immunology), ***Dr. Česlovas Venclovas,*** (Head of Laboratory of Bioinformatics), ***Prof. Virginijus Šikšnys,*** (Head of Laboratory of Protein-Nucleic Acids interactions).

Laboratory of Protein-Nucleic Acids Interaction

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Adriana Daškevič
Rūta Žalytė
Inga Ramonaite

Research overview

The overall research theme in our lab is the structural and functional characterization of enzymes and enzyme assemblies that contribute to the bacteria defense systems which target invading nucleic acids. In particularly, we are involved in the in deciphering structural and molecular mechanisms of restriction enzymes, and the molecular machinery involved in the CRISPR function. We are using X-ray crystallography, mutagenesis, and functional biochemical and biophysical assays to gain information on these systems.

Structure and function of restriction endonucleases.

Restriction and modification systems commonly act as the first line of intracellular defense against foreign DNA and function as sentries that guard bacterial cells against invasion by bacteriophage. R-M systems typically consist of two complementary enzymatic activities, namely restriction endonuclease (REase) and methyltransferase (MTase). In typical RM systems REase cuts foreign DNA but does not act on the host genome because target sites for REase are methylated by accompanying MTase. REases from 4000 bacteria species with nearly 330 differing specificities have been characterised. REases have now gained widespread application as indispensable tools for the in vitro manipulation and cloning of DNA. However, much less is known about how they achieve their function.

In the Laboratory of Protein-DNA Interactions we focus on the structural and molecular mechanisms of restriction enzymes. Among the questions being asked are: How do the restriction enzymes recognize the particular DNA sequence? What common structural principles exist among restriction enzymes that recognize related nucleotide sequences? How do the sequence recognition and catalysis are coupled in the function 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 (see below for the details).

Structure and molecular mechanisms of CRISPR/Cas systems.

Recently, an adaptive microbial immune system CRISPR (clustered regularly interspaced short palindromic repeats) has been identified that provides acquired immunity against viruses and plasmids. CRISPR represents a family of DNA repeats present in most bacterial and archaeal genomes. CRISPR loci usually consist of short and highly conserved DNA repeats, typically 21 to 48 bp, repeated from 2 to up to 250 times. The repeated sequences, typically specific to a given CRISPR locus, are interspaced by variable sequences of constant and similar length, called spacers, usually 20 to 58 bp. CRISPR repeat-spacer arrays are typically located in the direct vicinity of cas (CRISPR associated) genes. Cas genes constitute a large and heterogeneous gene family which encodes proteins that often carry functional nucleic-acid related domains such as nuclease, helicase, polymerase and nucleotide binding. The CRISPR/Cas system provides acquired resistance of the host cells against bacteriophages. In response to phage infection, some bacteria integrate new spacers that are derived from phage genomic sequences, which results in CRISPR-mediated phage resistance. Many mechanistic steps involved in invasive element recognition, such as novel repeat manufacturing, and spacer selection and integration into the CRISPR locus, remain uncharacterized (see below for the details).

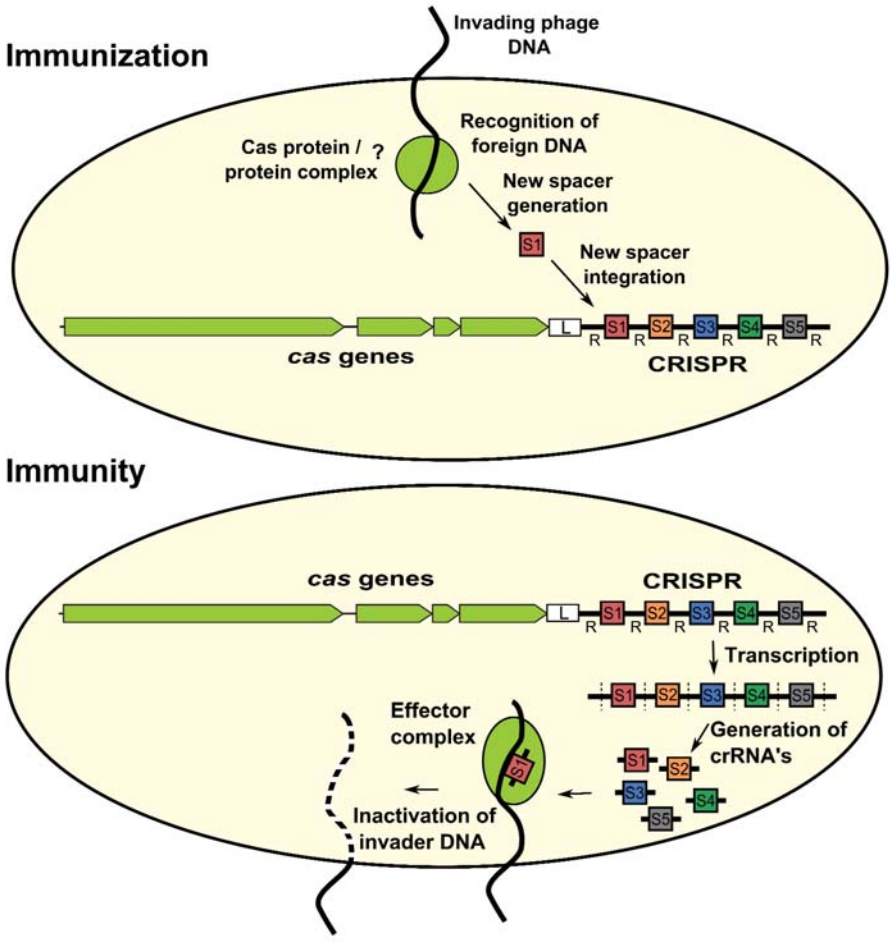

Figure 1. CRISPR/Cas system. CRISPR loci consist of short and highly conserved DNA repeats (R) interspaced by variable sequences of constant and similar length, called spacers (S). CRISPR repeat-spacer arrays are typically located in the direct vicinity of cas (CRISPR-associated) genes. In the immunisation steps, it is proposed that Cas proteins incorporate foreign DNA as new spacer sequences. This is a precise process that adds spacers of similar length to one end of the repeat. Thus, the repeat series acts as a historical record of viral infections. In the immunity step, it is proposed that RNA from the repeat region is produced and processed by Cas proteins to produce short signal RNAs. These crRNAs are then used to specifically target invading DNA for degradation.

Structure and function of restriction endonucleases: projects overview

DNA synapsis through transient tetramerization triggers cleavage by Ecl18kI restriction enzyme.

To cut DNA at their target sites, restriction enzymes assemble into different oligomeric structures. The Ecl18kI endonuclease in the crystal is arranged as a tetramer made of two dimers each bound to a DNA copy. However, free in solution Ecl18kI is a dimer. To find out whether the Ecl18kI dimer or tetramer represents the functionally important assembly, we generated mutants aimed at disrupting the putative dimer-dimer interface and analysed the functional properties of Ecl18kI and mutant variants. We show by atomic force microscopy that on two-site DNA, Ecl18kI loops out an intervening DNA fragment and forms a tetramer. Using the tethered particle motion technique, we demonstrate that in solution DNA looping is highly dynamic and involves a transient interaction between the two DNA-bound dimers. Furthermore, we show that Ecl18kI cleaves DNA in the synaptic complex much faster than when acting on a single recognition site. Contrary to Ecl18kI, the tetramerization interface mutant R174A binds DNA as a dimer, shows no DNA looping and is virtually inactive. We conclude that Ecl18kI follows the association model for the synaptic complex assembly in which it binds to the target site as a dimer and then associates into a transient tetrameric form to accomplish the cleavage reaction.

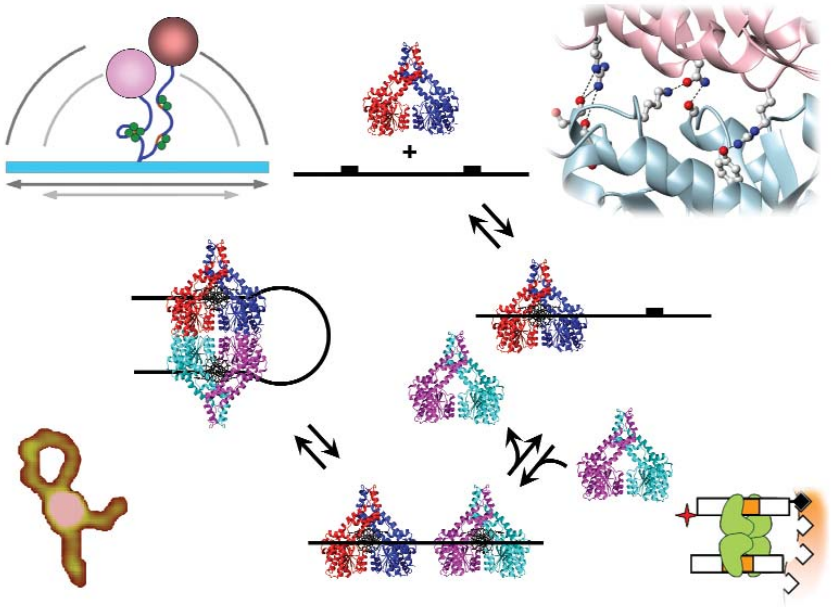

Figure 2. Reaction pathway of the Ecl18kI restriction enzyme on the two-site DNA. At Ecl18kI concentrations much below that of the DNA, a single Ecl18kI dimer presumably binds to only one individual target site. Binding of the second dimer at increased enzyme concentrations produces an unlooped protein-DNA complex, where two dimers act on the two DNA sites independently. Tetramerization of two DNA-bound Ecl18kI dimers results in the looped synaptic complex, which is optimal for catalysis and gives in fast cleavage.

A novel mechanism for the scission of double-stranded DNA: BfiI cuts both 3'-5' and 5'-3' strands by rotating a single active site.

Metal-dependent nucleases that generate double-strand breaks in DNA often possess two symmetrically-equivalent subunits, arranged so that the active sites from each subunit act on opposite DNA strands. Restriction endonuclease BfiI belongs to the phospholipase D (PLD) superfamily and does not require metal ions for DNA cleavage. It exists as a dimer but has at its subunit interface a single active site that acts sequentially on both DNA strands. The active site contains two identical histidines related by 2-fold symmetry, one from each subunit. This symmetrical arrangement raises two questions: first, what is the role and the contribution to catalysis of each His residue; secondly, how does a nuclease with a single active site cut two DNA strands of opposite polarities to generate a double-strand break. In this study, the roles of active-site histidines in catalysis were dissected by analysing heterodimeric variants of BfiI lacking the histidine in one subunit. These variants revealed a novel mechanism for the scission of double-stranded DNA, one that requires a single active site to not only switch between strands but also to switch its orientation on the DNA.

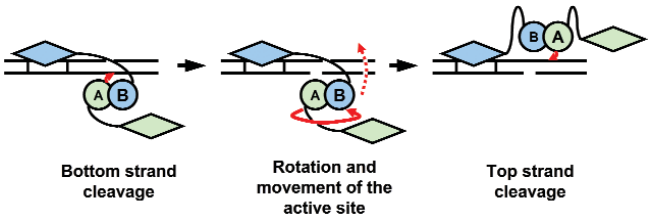

Figure 3. A model for the reactions of WT BfiI on the bottom and the top strand of a DNA duplex. The H105 residue from the same subunit of the homodimer, the 2° subunit not bound to the DNA makes the nucleophilic attacks on the target phosphodiester bonds in both bottom and top strands of the DNA. To match the anti-parallel orientation of the two strands, the N-terminal domains of BfiI must rotate by 180° between the two hydrolysis reactions.

Two-in-one: EcoRII restriction enzyme combines two radically different mechanisms to interact with the three target sites.

EcoRII restriction endonuclease is specific for the 5'-CCWGG sequence (W stands for A or T); however, it shows no activity on a single recognition site. To activate cleavage it requires binding of an additional target site as an allosteric effector. EcoRII dimer consists of three structural units: a central catalytic core, made from two copies of the C-terminal domain (EcoRII-C), and two N-terminal effector DNA binding domains (EcoRII-N). Here, we report DNA-bound EcoRII-N and EcoRII-C structures, which show that EcoRII combines two radically different structural mechanisms to interact with the effector and substrate DNA. The catalytic EcoRII-C dimer flips out the central T:A base pair and makes symmetric interactions with the CC:GG half-sites. The EcoRII-N effector domain monomer binds to the target site asymmetrically in a single defined orientation which is determined by specific hydrogen bonding and van der Waals interactions with the central T:A pair in the major groove. The EcoRII-N mode of the target site recognition is shared by the large class of higher plant transcription factors of the B3 superfamily.

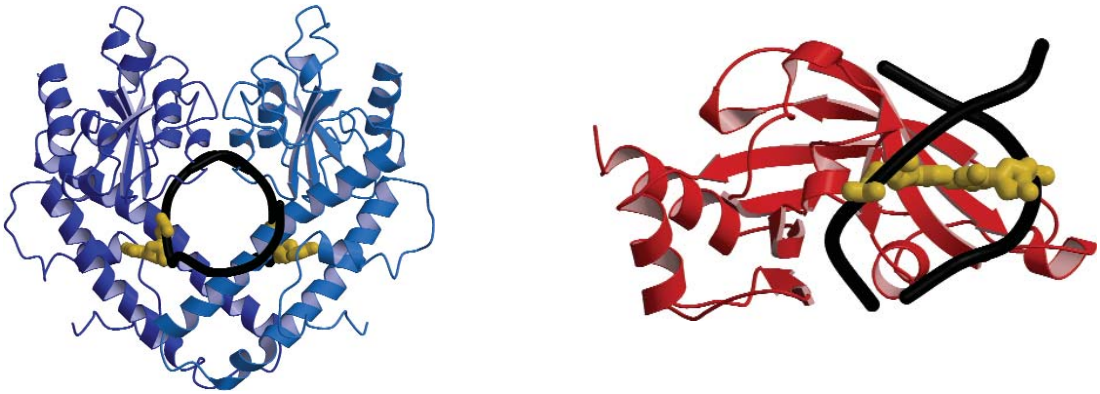

Figure 4. Crystal structure of EcoRII-C–DNA (A) and EcoRII-N–DNA (B) complexes.

Single-molecule dynamics of the DNA-EcoRII protein complexes revealed with high-speed atomic force microscopy.

The study of interactions of protein with DNA is important for gaining a fundamental understanding of how numerous biological processes occur, including recombination, transcription, repair, etc. In this study, we use the EcoRII restriction enzyme, which employs a three-site binding mechanism to catalyze cleavage of a single recognition site. Using high-speed atomic force microscopy (HS-AFM) to image single-molecule interactions in real time, we were able to observe binding, translocation, and dissociation mechanisms of the EcoRII protein. The results show that the protein can translocate along DNA to search for the specific binding site. Also, once specifically bound at a single site, the protein is capable of translocating along the DNA to locate the second specific binding site. Furthermore, two alternative modes of dissociation of the EcoRII protein from the loop structure were observed, which result in the protein stably bound as monomers to two sites or bound to a single site as a dimer. From these observations, we propose a model in which this pathway is involved in the formation and dynamics of a catalytically active three-site complex.

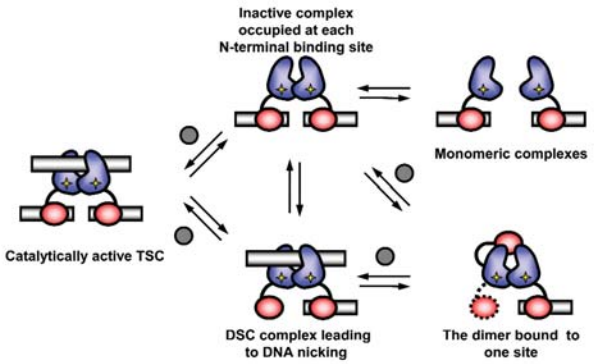

Figure 5. Dynamic model of the catalytically active triple synaptic complex (TSC) of EcoRII. Rectangles in this scheme denote the DNA capable of binding to one of the three binding sites of the protein. DNA binding sites at the N-and C-terminal domains are shown in red and blue, respectively. An active TSC complex is formed after the two N-terminal binding sites are occupied, and the third strand binds to C-terminal pocket. The TSC complex breaks apart by the dissociation into two monomeric complexes or a dimeric complex bound to a single binding site.

Time-resolved fluorescence studies of nucleotide flipping by restriction enzymes.

Restriction enzymes Ecl18kI, PspGI and EcoRII-C, specific for interrupted 5-bp target sequences, flip the central base pair of these sequences into their protein pockets to facilitate sequence recognition and adjust the DNA cleavage pattern. We have used time-resolved fluorescence spectroscopy of 2-aminopurine-labelled DNA in complex with each of these enzymes in solution to explore the nucleotide flipping mechanism and to obtain a detailed picture of the molecular environment of the extrahelical bases. We also report the first study of the 7-bp cutter, PfoI, whose recognition sequence (T/CCNGGA) overlaps with that of the Ecl18kI-type enzymes, and for which the crystal structure is unknown. The time-resolved fluorescence experiments reveal that PfoI also uses base flipping as part of its DNA recognition mechanism and that the extrahelical bases are captured by PfoI in binding pockets whose structures are quite different to those of the structurally characterized enzymes Ecl18kI, PspGI and EcoRII-C. The fluorescence decay parameters of all the enzyme-DNA complexes are interpreted to provide insight into the mechanisms used by these four restriction enzymes to flip and recognize bases and the relationship between nucleotide flipping and DNA cleavage.

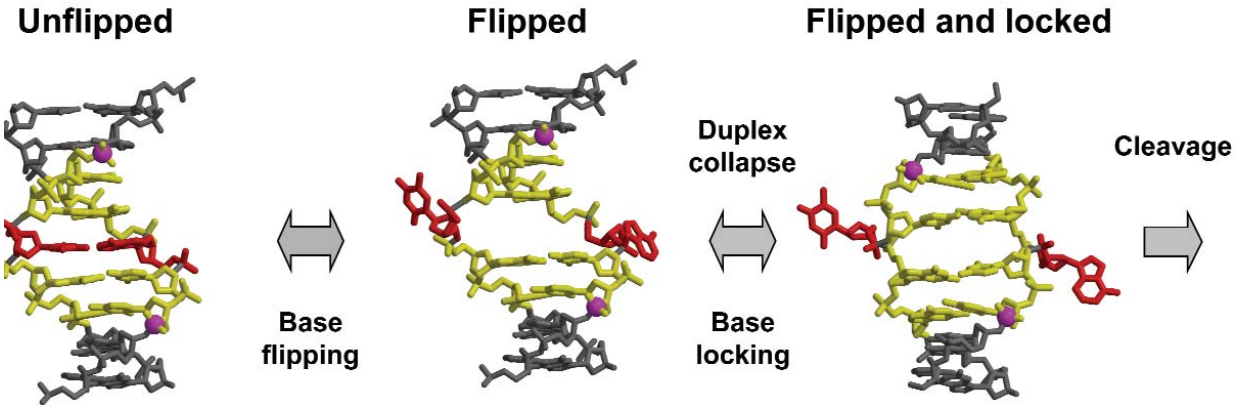

Figure 6. DNA conformational changes on the reaction pathway of the nucleotide flipping restriction enzymes. The target base can be unflipped (as largely seen in the Ecl18kI W61A complex), in a highly dynamic flipped (but not locked) state (predominant in the PspGI complex) or locked in the enzyme's flipping pocket (as in wt Ecl18kI and EcoRII-C complexes).

Structure and molecular mechanisms of CRISPR/Cas systems: projects overview

Streptococcus thermophilus DGCC7710 strain, for which biological activity of the CRISPR/Cas system has been directly demonstrated in a phage challenge assay, contains four distinct systems: CRISPR1, CRISPR2, CRISPR3 and CRISPR4. Direct spacer incorporation activity has been demonstrated for the CRISPR1 and CRISPR3 systems, with the former being more active. The CRISPR2 system seems to be disrupted and non-functional, whilst functional activity of CRISPR4 has not yet been demonstrated. Cas genes, which are specific to the repeat regions and fall into different families, are located in close proximity to the spacer-repeat region and encode proteins that often carry functional nucleic-acid related domains such as nucleases, helicases, polymerases and nucleotide binding. We aim to characterize the functional and biochemical activities of Cas proteins belonging to the CRISPR1, CRISPR3 and CRISPR4 systems of *S. thermophilus*.

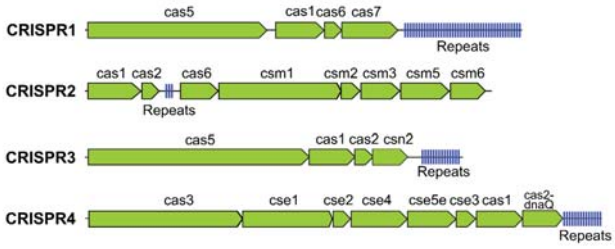

Figure 7. CRISPR/Cas systems of S. thermophilus DGCC7710. Cas protein clusters of the CRISPR1 and CRISPR3 systems belong to the Nmeni subtype whilst CRISPR4 belongs to the Ecoli subtype.

Collaboration

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Dr. P. Horvath, DANISCO, France

Prof. Dr. Y. Lyubchenko, University of Nebraska Medical Centre

Prof. Dr. A. Pingoud, University of Giessen, Germany

Prof. Dr. M. Szczelkun, Bristol University, UK

Prof. Dr. G. Wuite, Vrije Universiteit, the Netherlands

Grants

EC Framework 6 Programme

The Wellcome Trust

The Royal Society

Lithuanian State Science and Studies Foundation

Research Council of Lithuania

Selected Publications 2008-2010

1. **Gasiunas G., Sasnauskas G., Tamulaitis G.,** Claus Urbanke, Razaniene D., and **Siksnys V.** Tetrameric restriction enzymes: expansion to the GIY-YIG nuclease family. Nucleic Acids Res. 2008, 36(3):938-49.
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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 (SAM or AdoMet) to N-, C-, O- or S-nucleophiles in DNA, RNA, proteins or small biomolecules [8, 9].

In DNA, enzymatic methylation of nucleobases serves to expand the information content of the genome in organisms ranging from bacteria to mammals. Postreplicative methylation of the genome is accomplished by DNA methyltransferases. 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. Besides 5-methylcytosine, another modification state of cytosine, 5-hydroxymethylcytosine (hmC), has recently been (re)discovered in mammalian DNA. Aberrant DNA modification patterns are known to correlate 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 biological DNA modifications 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 DNA cytosine-5 methyltransferase (M.HhaI, recognition target GCGC) 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

D. Daujotytė, R. Gerasimaitė, E. Merkienė, Z. Staševskij, M. Tomkuvienė, Z. Liutkevičiūtė

Enzymatic DNA cytosine-5 methylation is a complex reaction that proceeds via multiple steps such as binding of cofactor AdoMet and substrate DNA, rotation of the target cytosine out of the DNA helix (base flipping), conformational rearrangement of the mobile catalytic loop, activation of the target cytosine via formation of a transient covalent bond and transfer of the methyl group from the bound cofactor onto the target cytosine. Despite of extensive studies of the model HhaI methyltransferase, initial events in the base flipping mechanism of this model enzyme remained elusive [10,11]. We use mutagenesis, biochemical analysis, enzyme kinetics, NMR and fluorescence spectroscopy, x-ray crystalography to delineate the elementary steps on the reaction pathway of HhaI MTase (Klimašauskas et al., EMBO J. 1998, 17: 317-324; Serva et al., Nucleic Acids Res. 1998, 26: 3473-3479; Vilkaitis et al., J. Biol. Chem. 2001, 276: 20924-20934; Daujotytė et al., Structure, 2004, 12: 1047-1055; Merkienė and Klimašauskas, Nucleic Acids Res., 2005, 33: 307-315; Neely et al., Nucleic Acids Res., 2005, 33: 6953-6960). Our recent 'snapshot pictures' from a series of new crystal structures (in collaboration with the group of Saulius Gražulis at the Laboratory of Protein – DNA Interactions) of 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). Using stopped flow kinetic analysis we have recently been able to directly follow, in a chemically unperturbed system, the target base flipping and its covalent activation. Combined with studies of M.HhaI variants containing redesigned tryptophan fluorophores, our experiments show that the target base flipping and the closure of the mobile catalytic loop occur simultaneously. Subsequently, the covalent activation of the target cytosine is closely followed by but is not coincident with the methyl group transfer from the bound cofactor. These findings provide new insights into the time resolved steps of this physiologically important reaction mechanism and pave the way to in-depth studies of other base-flipping systems (in preparation).

DNA base flipping, first demonstrated in a crystal structure of the M.HhaI–DNA complex, now is also known to be used by a wide variety of DNA enzymes [10,11]. However, no simple and reliable technique for the detection of the natural nucleobase extrahelical cytosines is available. We have recently demonstrated the first application of 2-chloracetaldehyde for chemical detection of 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 [1].

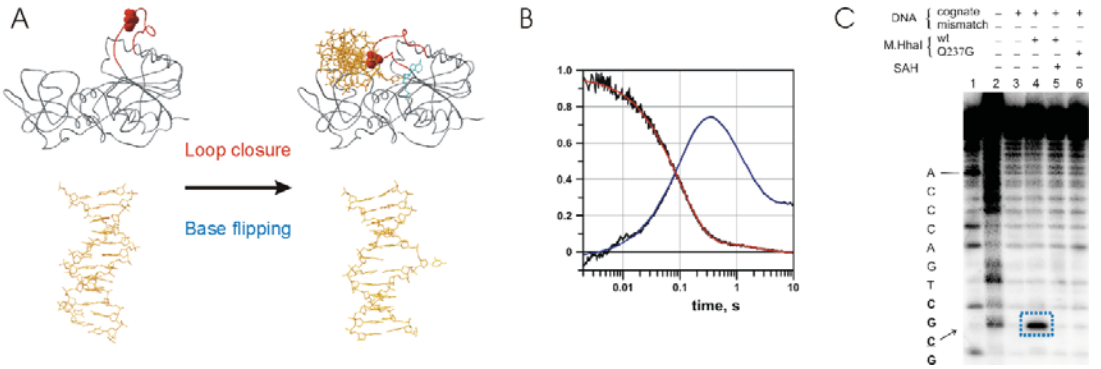

Figure 1. Pre-catalytic conformational rearrangements in a DNA cytosine-5 methyltransferase, M.HhaI. (A) - Catalytic loop closure and flipping of the target cytosine out of the DNA helix [10, 11]. Protein is shown as backbone trace, built-in fluorogenic Trp residue is shown in red space-fill and DNA is shown in orange stick representation. (B) - Real-time observation of the target cytosine flipping (blue trace - hyperchromicity change) and the catalytic loop motion (red trace, fluorescence of built-in Trp fluorophore) using stopped-flow technique [in preparation]. (C) - Chemical detection of flipped out cytosine bases in M.HhaI-DNA complexes using chloroacetaldehyde modification [1].

HPLC-MS analysis performed by Zita Liutkeviciute

Engineering sequence specificity of DNA methyltransferases

Structure-guided rational protein design combined with random mutagenesis and selection was used to change the sequence specificity of the HhaI MTase from GCGC to GCG [3]. The specificity change was brought about by a five-residue deletion and introduction of two arginine residues within and nearby one of the target recognizing loops in the enzyme. DNA protection assays, bisulfite sequencing and enzyme kinetics showed that the best selected variant is comparable to wild-type M.HhaI in terms of sequence fidelity and methylation efficiency, and supersedes the parent enzyme in transalkylation of DNA using synthetic cofactor analogs. The designed C5-MTase can be used to produce hemimethylated CpG sites in DNA, which are valuable substrates for studies of mammalian maintenance MTases.

Engineering the catalytic reaction of methyltransferases for targeted covalent labeling of biopolymers

G. Lukinavičius, V. Masevičius, G. Urbanavičiūtė, R. Gerasimaitė,

In collaboration with the laboratory of Prof. Elmar Weinhold (RWTH Aachen, Germany), we 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 and demonstrated that allylic and propargylic side chains can be efficiently transferred by DNA MTases with high sequence- and base-specificity (Dalhoff et al, Nature Chem. Biol., 2006, 2: 31-32; Klimašauskas and Weinhold, Trends Biotechnol., 2007, 25: 99-104).

Using DNA MTases along with their novel cofactors that carry useful functional (amino, alkyne, thiol) or reporter (biotin) groups, we demonstrated that our new approach name mTAG (methyltransferase-directed Transfer of Activated Groups) can be used for efficient sequence-specific functionalization and labeling of a wide variety of model and natural DNA substrates (Lukinavicius et al., J. Amer. Chem. Soc., 2007, 129: 2758-2759 and in preparation). For example, we have used mTAG DNA labeling, followed by molecular combing of DNA onto a polymer-coated surface, to achieve sub-diffraction limit positioning of the sequence-specifically attached fluorophores in single DNA molecules (in collaboration with R.K. Neely and J.Hofkens, KU Leuven, Belgium). The result is a 'DNA fluorocode'; a linear description of the DNA sequence that can be read and analyzed like a barcode. We have demonstrated the generation of a fluorocode for genomic DNA from the lambda phage virus using M.HhaI [6]. Individual fluorophores are positioned to within 200 base pairs of their predicted position on a reference map of the genome. The fluorocode can be generated from a handful of molecules and entirely independently of any reference sequence. These findings envision numerous applications of the new labeling technique in functional studies, DNA-based nanotechnologies and medical diagnostics. Moreover, we and our collaborators find that the newly developed cofactors can be used for targeted transfer of functional groups or other chemical entities to RNA and proteins using appropriate MTases as catalysts (in preparation).

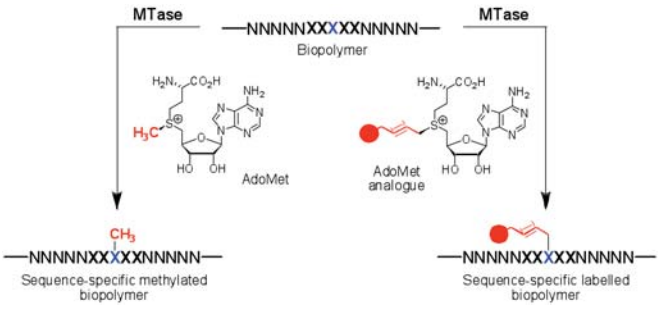

Figure 2. Sequence-specific covalent modification of biopolymers (N and X = nucleotide pairs for DNA and nucleotides for RNA or amino acids for proteins, XXXXX = recognition sequence of the MTase, X = target nucleotide (pair) or amino acid) catalyzed by a large number of DNA, RNA or protein methyltransferase (MTase). Left. Biological methylations involving the AdoMet cofactor. Right: methyltransferase-direct Transfer of Activated Groups (mTAG) carrying a reactive or reporter functionality (red sphere) from a double-activated AdoMet analog onto a target nucleobase in DNA.

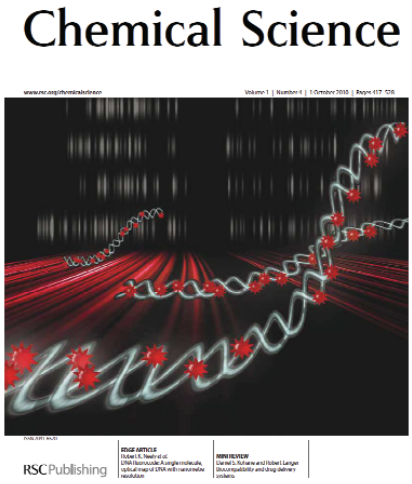

Figure 3. 'DNA fluorocode' (illustration from the back cover of Chemical Science, 2010, vol. 1, issue 4.). Linear physical positioning of fluorophores (shown as white elongated vertical 'bars') via sequence-specific methyltransferase-directed labeling of bacteriophage DNA followed by sub-diffraction resolution imaging of fluorophores in surface-combed individual DNA molecules. Such a linear description of the DNA sequence can be read and analyzed like a barcode [6].

Novel non-cofactor reactions of DNA methyltransferases

Z. Liutkevičiūtė, V. Masevičius, G. Lukinavičius, D. Daujotytė

Enzymatic transmethylations generally proceed via a direct nucleophilic attack of a target atom onto the sulfonium-bound methyl group of AdoMet. DNA cytosine-5 MTases use a covalent mechanism for nucleophilic activation of their target cytosine residues. To explore the chemical reactivity of the activated covalent intermediate, binary MTase-DNA complexes were screened against a series of electrophilic compounds such as aldehydes, ketones and electronegatively substituted vinyls. Notably, we have found that several DNA MTases catalyze covalent addition of exogenous aliphatic aldehydes to their target residues in DNA, yielding corresponding 5-α-hydroxyalkylcytosines [2]. To our knowledge, this is the first demonstration of wild-type cofactor-dependent enzymes catalyzing an atypical chemical reaction using non-cofactor-like exogenous substrates. The reactive aldehydes are not bona fide cofactors of SAM-dependent MTases because they lack an anchor moiety (such as adenosyl) that would assist in the formation of a discrete, specific complex with the enzyme.

We also showed that C5-MTases can promote the reverse reaction – the removal of formaldehyde from hmC [2]. Certain DNA repair enzymes can reverse alkylation damage via enzymatic oxidation of N-alkylated nucleobases to corresponding α-hydroxyalkyl derivatives, which spontaneously release an aldehyde from the ring nitrogen to generate the unmodified base. The enzymology of demethylation of genomic 5-methylcytosine remains elusive and highly debated; among others, a similar two-step route has been proposed based on the above examples and preliminary observations. Our findings offer a direct demonstration of an enzymatic hmC-to-cytosine conversion, thus providing a plausible chemical precedent for an oxidative mechanism of DNA demethylation in mammalian DNA.

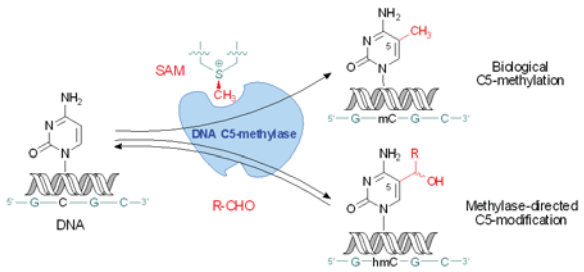

Figure 4. Transformations of cytosine in DNA catalyzed by C5-MTases. Biological methylations by C5-MTases occur via direct transfer of the methyl group from cofactor SAM onto a target cytosine residue in DNA yielding 5-methylcytosine (mC, top route). In the absence of SAM, C5-MTases can catalyze nucleophilic addition of exogenous aldehydes (bottom route) to the target cytosine to give corresponding 5-(α-hydroxyalkyl)cytosines (reaction with formaldehyde yields 5-hydroxymethylcytpsine, hmC). This modification is reversed back to unmodified DNA by the enzyme in the absence of the exogenous aldehyde [2]. Modifying reagents are shown in red, C5-MTase is depicted in blue.

Molecular tools for epigenome profiling

E. Kriukiene, Z. Liutkevičiūtė, G. Urbanavičiūtė, Z. Staševskij

Over the last decade, epigenetic phenomena have claimed a central role in the research of cell regulatory processes. In addition, there is increasing evidence that epigenetic factors may play a significant role in various human diseases. One of the best understood epigenetic mechanisms is DNA methylation. In the mammalian genome, cytosines (C) are known to exist in two functional states: unmethylated or methylated at the 5-position of the pyrimidine ring (5mC). Cytosines followed by guanine (CpG dinucleotides) are the preferred targets for methylation. Recent studies of genomic DNA from the mouse brain, neurons and embryonic stem cells detected evidence that CG sequences also contain 5-hydroxymethylcytosine (hmC). As their interactions with cellular proteins are distinct, the 5-hydroxymethyl groups in DNA likely play an independent role in yet unknown epigenetic regulation of embryonic development, brain function, and cancer progression. However, further studies of these intriguing phenomena are hampered by the lack of adequate analytical techniques. Numerous methods have been developed over the past several years to investigate DNA methylation profiles across genomes. However, all those methods are binary - i.e. designed to distinguish only the two epigenetic states of cytosine: methylated versus unmodified.

We aim to develop new experimental approaches to genome-wide profiling of DNA modifications for epigenome studies and improved diagnostics. One such strategy is based on selective mTAG labeling and enrichment of unmethylated CpG sites in the genome (note that methylated target sites cannot be labeled) followed by analysis of the enriched fractions on tiling microarrays (in collaboration with Prof. Art Petronis, CAMH, Canada). A substantial effort is also dedicated to the development of new technologies that are capable of mapping of hmC in mammalian genomes based on known and the newly discovered enzymatic transformations of the hydroxylmethyl group in hmC [2, 13-15]. Combining these novel approaches with DNA microarray analyses and next generation sequencing, we will study epigenome-wide distribution of hmC to unveil the intra- and inter-individual variation of this novel cytosine modification in DNA.

Mechanism of HEN1-mediated small RNA methylation

G. Vilkaitis, A. Plotnikova, S. Jachimovičiūtė

Small RNAs such as miRNAs, siRNAs and piRNAs are essential for post-transcriptional gene regulation in eukaryotic organisms including humans. Biogenesis of plant miRNAs and siRNAs or animal piRNAs and Ago2-loaded siRNAs involves modification of small RNA molecules at the 2'-O group of 3'-termini. The paradigm of the small RNA 2'-O-methyltransferases family - large multidomain methyltransferase HEN1 from Arabidopsis thaliana catalyzes methyl group transfer to miRNA/miRNA* and siRNA/siRNA*. The methylation is crucial for plant small RNA stability in vivo since the accumulation of miRNAs and ta-siRNAs in hen1 mutants is greatly reduced. A number of molecular and biochemical approaches have been developed in our laboratory along with the group of Prof. Xuemei Chen (UC Riverside, USA) to examining the unique methyltransferase HEN1. We have found that, unlike its homologs from animals and Drosophila which methylate single-stranded RNA substrates in vitro, HEN1 displays a strong preference toward duplex RNAs. Studies of HEN1-RNA interaction, pre-steady-state and steady-state kinetics provided important insights into substrate interactions and contributions of individual steps during catalysis by HEN1 [7]. Our findings suggest an important role for the N-terminal domains in stabilizing the catalytic complex, and indicate that major structural determinants required for selective recognition and methylation of RNA duplexes reside in the C-terminal domain.

Archaeal C/D box RNPs: towards programmable sequence-specific labeling of RNA

M. Tomkuvienė, G. Lukinavičius

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 they can be synthetically 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 of RNA, which would provide a new labeling technology with broad applicability. In collaboration with Dr. Beatrice Clouet d'Orval at Université Paul Sabatier, we have in vitro reconstituted such sRNP from a thermophilic (Pyrococcus abyssi) and mesophilic (Methanobrevibacter smithii) archaeal species. 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.

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Selected publications 2008-2010

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* recommended by Faculty of 1000 Biology

** Edge Article, commentary in Highlights Chem. Sci. http://www.rsc.org/Publishing/ChemScience/Volume/2010/09/visualising_dna.asp

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Humanization of yeast Saccharomyces cerevisiae

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The purpose of this project is to generate yeast cell lines carrying integrated human genes of early stages of cell secretion pathway, capable to effectively synthesize secreted mammalian proteins and ensure for their correct folding in yeast cells. We offer the scheme for selecting of suitable human proteins and finding of their optimal combinations, resulting in most effective synthesis of active recombinant biomedical proteins in yeast. It is based on the results of our previous works including original yeast expression systems developed in our laboratory, usage of a convenient gene-reporter and elucidation of molecular processes leading to formation of insoluble aggregates of recombinant model glycoproteins in yeast cells. The transfer of human genes into yeast genome will be combined with inactivation of yeast genes, responsible for yeast-specific block of recombinant protein maturation at early stages of cell secretory pathway and secretion lowering in yeasts. Proteomic analysis of the processes in genetically re-engineered yeast enables us to monitor targeted humanization of cell secretory pathway and choose optimal solution. Humanized yeast strains (carrying combinations of functional human genes in their genomes) could be directly used for generation of active biomedical proteins, firstly for the production of human virus surface glycoproteins. It would simplify the development of effective and uncostly recombinant vaccines and diagnostic reagents. Humanized yeast expression system should also be useful for production of important therapeutic proteins. Intended investigation should let us to answer the question which proteins in human/mammalian cell secretion pathway are responsible for correct processing and folding of complex secreted transmembrane protein precursors. Lower eukaryotic expression systems such as yeast and insect cells are typically unable to effectively synthesize active human proteins of this type; the recombinant products are usually incorrectly folded and inactive. Elucidation of the components that determine these differences between cells of higher and lower eukaryotes would provide important data concerning action of molecular mechanisms in eukaryotic cells and evolution of the cells in complex multicellular organisms.

The project involves interdisciplinary research in fields of proteomics, genetic engineering, recombinant protein production, protein folding, molecular interactions, cell biology, systems biology and biomedicine. The aims of the project are to:

1. Develop yeast strains carrying integrated human genes of cell secretion pathway, ensuring effective synthesis of secreted human biomedical proteins and their correct folding in yeast cells. 2. Employ developed humanized yeast strains for the production of recombinant biopharmaceutical proteins.

During the first two years of the project such human genes as HSP71, SRP54, SRP9, TRAM1, Sec61α izoformos S61A1 and S61A2, GRP78-BiP, PDIA1, CALR, CANX, ERp57 SEC61B, SEC61G, HSP90B1, UGCGL1, SRP14, SRP19, SRP68, SRP72, SRPR, SRPRB ir EEF1A1 were cloned and expression was confirmed by the detection of of corresponding human proteins in yeast. Constructed yeast strains were used for co-expression experiments of viral glycoproteins. We demonstrated that some human genes significantly enhanced the yield of viral glycoprotein and its solubility when expressed in humanized yeast.

Development of system for gene introduction, multiplication and stable maintanance in yeast using subtelomeric regions.

The project is funded from the postdoctoral fellowship to Dr. E. Astromskas which is supplied by the "Postdoctoral Fellowship Implementation in Lithuania" project (2010-2011). The aim of the project is to create a novel system for introduction genes into yeast genome and multiply them to overexpress protein of interest. The system will be based on the Y' subtelomeric regions of Saccharomyces cerevisiae yeast. These Y' repeats get amplified to a big extent after losing telomerase activity due to recombination. Thus, our aim is to introduce number of human genes and overexpress them in vivo using this method. The target genes are taken from the yeast humanization project, in order to create a stable system for overexpressing human chaperones in yeast and to understand protein folding for number of glycosylated proteins.

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 mimic 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 for induction of humoral response.

In addition to the humoral immunity, we have demonstrated that HaPyV VP1 VLPs were able to induce a T-cell immune response against inserted human HLA-*A2-restricted mucin 1 CTL tumor epitope (MUC1). Yeast-expressed VP1-MUC1 VLPs were tested for their interaction with human monocyte-derived dendritic cells and the induction of an T-cell response against inserted MUC1 peptide in vitro. Induction of maturation of the dendritic cells was evidenced by increased levels of surface maturation markers and a reduced uptake of FITC dextran and Lucifer Yellow. HaPyV-VP1-derived VLPs with MUC1 peptide at specific sites of VP1 preserved original features of entry and potential to induce human DCs (hDCs) maturation confirming the valuable insertion capacity of the VP1 carrier. Moreover, the dendritic cells stimulated with these VLPs produced interleukin-12 and stimulated MUC1 specific CD8-positive T-cell responses in vitro. In addition, several human cancer cell lines were demonstrated to stain positive with the epitope-specific antibodies generated against these chimeric VLPs. (Dorn et. al., 2008).

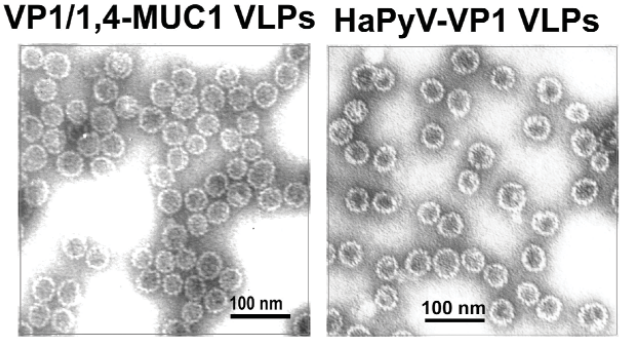

Figure 1. EM of virus-like particles (VLPs)

In conclusion, the dual potential of MUC1-harboring VLPs in inducing both a humoral and cytotoxic immune response make them an attractive adjunct to traditional cytotoxic agents for tumor eradication. HaPyV VP1-derived VLPs may represent promising vaccines not only for viral infections but also for cancer and auto-immune diseases as are successfully employed for hybridoma technology.

Development of diagnostic tools for Merkel cell polyoma virus

The project was supported by the Lithuanian State Science and Studies Foundation grant N-09005 in 2009 and by Research Council of Lithuania in 2010.

Merkel cell carcinoma (MCC) is rare but very aggressive skin cancer. The number of cases of this disease is increasing and the prognosis for MCC patients depends greatly on the stage of the disease at the time of diagnosis. Merkel cell polyoma virus (MCPyV) was found in 2008 and it was identified as etiologic agent of human Merkel cell carcinoma. MCC diagnostics in Lithuania is based on clinical symptoms and MCPyV was never investigated as MCC etiologic agent. The project aims to generate the recombinant MCPyV capsid protein and develop modern assays for molecular diagnostics and immunodiagnostics of MCPyV infections in Merkel carcinomas.

The synthetic MCPyV VP1 gene was cloned in yeast expression system and MCPyV VP1 virus like particles (VLPs) were purified. It was shown that after immunization of mice MCPyV VP1 VLPs activated B and T cells and induced humoral and cellular immune response. 9G6, 24D11 11A2 monoclonal antibodies recognizing MCPyV VP1 VLPs were generated and 11A2 were successfully used for MCPyV diagnostics in immunohistochemical assay using 2 clinical MCC samples. PCR-based assay was positive for 5 clinical MCC samples using 4 pairs of primers and sequencing of obtained DNA fragments confirmed presence of MCPyV in these samples. The KI polyoma virus VP1 protein encoding gene was cloned and expressed in yeast also. For investigation of the prevalence of MCPyV and other human polyoma viruses in Lithuania the MCPyV, KIPyV, JCV, BKPyV, SV40 VP1 VLPs were used for serological assays.

Engagement of metagenomic analysis of extremophile viruses from hot underground waters of Lithuania searching for the new enzymes

The project was supported 2007-2009 by the Lithuanian State Science and Studies Foundation grant N-07005

Microorganisms that survive in the extreme environments are of great interest, because they can serve as a source of enzymes having unique properties. Identification and cultivation of unknown viruses directly from the environment is complicated but metagenomic analyses of uncultured viral communities can provide insights into the composition and structure of environmental viral communities and are wellspring of novel sequences and new genes.

The aim of this study was to search for the new enzymes of viruses from the extreme environment. We examined the variety of bacterial viruses present in natural hot underground waters of Lithuania and artificial overground chilled water of thermal power-station enginery, waste water treatment reservoirs (thermal power-station of Elektrenai) and urban effluent water of Vilnius were examined. The results of the electron microscopy revealed that tailed phages belonging to the order Caudovirales were predominant in all samples tested. Based on the shape and morphology these phages could be assigned to the virus families Siphoviridae, Myoviridae and Podoviridae. Viral particles with apparent morphology of the Lipotrixviridae, Tectiviridae and Inoviridae families were also observed. Although the DNA extracted from the viral fraction from all sources and amplified using phi29 DNA polymerase was mostly soil and water ecosystems bacteria-derived some of the samples of DNA shared sequence similarities with fragments of genes of phages as Thermus phage phiYS40 which was extracted and enriched from DNA samples collected from the chilled water of thermal power-station enginery samples.

Viral particles with apparent morphology of the Myoviridae, Siphoviridae, Inoviridae and Tectiviridae families were detected by electron microscopy of the thermophiles-enriched samples. The viral fraction of DNA isolated from the enriched samples appeared to encode ORF`s (or their segments), that shared sequence relatedness with Geobacillus, Clostridium, Heliobacterium, Listeria, Bacillus and Staphylococcus phage or prophage proteins. A fair amount of the ORF`s mentioned showed a certain degree of amino acid sequence identity with various proteins of Clostridium botulinum phage c-st or Bacillus subtilis phage SPBc2. Among the DNA extracted from the samples that were enriched at 60-65ºC and 55ºC, the reverse transcriptase, lisozyme and adenine- or cytosine-specific DNA methylase and DNA polymerase genes (or gene fragments) were detected. A bioinformatics analysis of several predicted ORF`s with unknown function identified two more possible candidates potentially useful in biotechnology: the viral site-specific endonuclease and DNA polymerase.

Two lytic Geobacillus staerothermophilus phages 2-11-1 and 2-11-2 with the double-stranded DNA genomes of ~40 and 55 kbp, respectively, as well as temperate Geobacillus bacteriophage 2KT-1 were isolated. The complete sequences for the lysozyme gene of these phages 2-11-2 and 2KT-1 were determined, and the gene for the lysozyme of 2KT-1 was cloned and overexpressed in E. coli. Bacteriophages belonging to the T4-type and smaller E. coli myoviruses that prefer higher temperature for growth (~48ºC) were also isolated. The complete or partial sequences for DNA polymerase as well as DNA and RNA ligase genes of these phages were determined. The gene for the DNA ligase of bacteriophage FV3, showing 56% of amino acid sequence identity with the DNA ligase orthologue of phage RV5, was cloned and overexpressed in E. coli.

Expression of glycoproteins of mumps and measles viruses in yeast

Proteomic approach was applied 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.

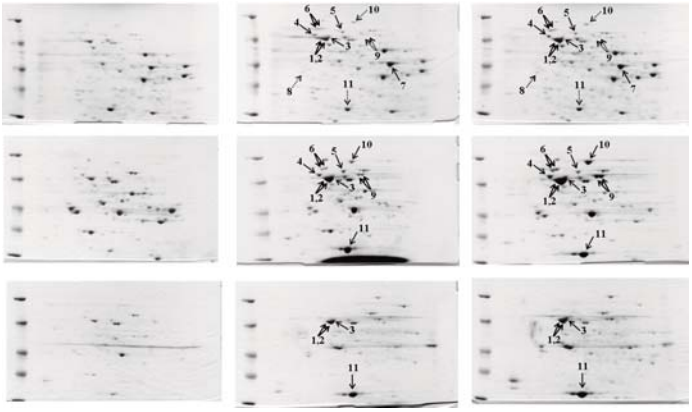

Figure 2. 2D gel electrophoresis of yeast proteins.

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.

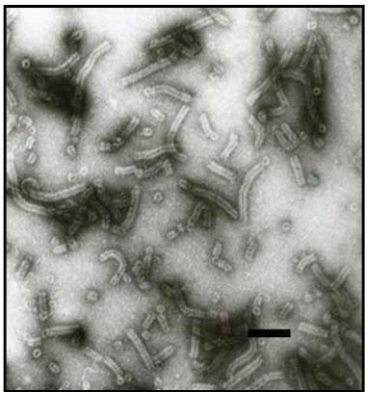

Figure 3. EM of recombinant human parainfluenza virus 1 and 3 nucleocapsid proteins in yeast Saccharomyces cerevisiae (Juozapaitis M. et al., 2007)

Technology development of long-acting recombinant proteins of therapeutic value

The project was supported by the Lithuanian State Science and Studies Foundation and UAB Profarma, grant No. N-07006 for 2007-2009.

Innovative process was proposed for the production of long-acting (prolonged) form of human recombinant proteins such as granulocyte colony stimulating factor (GCSF). The essential element of this project reports to linear dimer 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 mutants of monomer recombinant proteins of therapeutic value (Zaveckas M. et al., 2007).

Clearance rate experimental data of the developed GCSF dimer proteins showed its prolongation for several times in comparison with clearance of homologous monomer of GSCF protein. Constructed proteins are expected to compete with recombinant therapeutic proteins possessing prolonged activity in current markets. Linear dimers of the target protein obtained according the developed process to highly purified state and have been characterized by a set of analytical methods and tested for prolongation effect of their bioactivity and clearance rate on the models in vitro (cell culture) and in vivo (rats). The most potent selected protein candidates have been overpassed through further optimization to develop lab-scale production technology. Prepared technology has been addressed to the patent application procedure, with further perspective for licensing of this technology and implementation for industrial pharmaceutical production.

Development of technology of proteins (antibodies) neutralizing bacterial cytolysins with therapeutic and diagnostic use

The project was supported by the Lithuanian State Science and Studies Foundation and UAB Profarma, grant No. N-07006 and PBT-01/2010 for 2008-2010.

Main goal of the project was to prepare neutralizing monoclonal antibodies (Mabs) and their recombinant fragments against Gardnerella vaginalis hemolysin (vaginolizin) as well as against the other cholesterol-dependent bacterial cytolysins. Project was also aimed to the isolation of constructed antibodies or their fragments for testing as therapeutic drug or testing as diagnostic tool. Project included the selection of most potent single chain variable fragments (scFv) derived from cloned immunoglobulin cDNA for further development of purification technology of such recombinant proteins with potential therapeutic aplication.

During the project new member of cholesterol-binding family of cytolysins from Gardnerella species have been cloned (vaginolizin), expressed and purified as initial material for development of specific Mabs. In parallel, the other members of the same cytolysin family have been purified and tested. Neutralizing monoclonal antibodies against the vaginolizin have been constructed and experimentally tested applying clinical samples of Gardnerella. Specific Mabs suitable for diagnostic purposes have been selected. The single-chain variable fragments (scFv) from selected Mabs with toxin neutralizing activity were successively cloned, modified and expressed in E. coli bacteria. The obtained scFv have been tested for the best affinity to vaginolizin and other cytolysins. The lab-scale technology developed for selected scFv proteins with the highest neutralizing affinity. Patent applications for developed technologies applicable in diagnostic and therapeutic use have been issued.

Hantavirus vaccine and diagnostic tools

New methods for diagnostics of hantavirus

We evaluated an indirect and capture enzyme linked immunosorbent (ELISA) assays for detection of hantavirus specific immunoglobulins IgG and IgM in human serum samples using recombinant yeast-expressed hantavirus nucleocapsid (N) protein of Hantaan virus Fojnica strain. After establishment of the optimal conditions of the different tests the sensitivity and specificity of the new assays were determined.

For that purpose 93 serum samples were tested by indirect and capture assays. The level of IgG and IgM antibodies was measured. The serum specimens tests results were compared with commercially available Progen Hantavirus IgG and IgM detection kit (Heidelberg, Germany). By comparison serum results with the Progen kit the sensitivity of IgG and IgM indirect ELISA assays was 100%, the specificity 97.3% for IgM test and 100% for IgG test. Comparing the capture assay with Progen kit results the sensitivity was 100% for IgM assay and 80% for IgG assay. The specificity of IgM capture assay was 97.3% and 95% of IgG capture assay. In summary, the results of the tests using recombinant protein were concordant with Progen test and the yeast expressed N protein is suitable for hantaan diagnostics.

Characterization of monoclonal antibodies against hantavirus nucleocapsid protein and their use for immunohistochemistry on rodent and human samples

Monoclonal antibodies are important tools for various applications in hantavirus diagnostics. Recently we have generated Puumala virus (PUUV)-reactive monoclonal antibodies (mAbs) by immunization of mice with chimeric polyomavirus-derived virus-like particles (VLPs) harboring the 120 amino acids-long amino-terminal region of PUUV nucleocapsid (N) protein. Here we describe the generation of two mAbs by co-immunisation of mice with hexahistidine-tagged full-length N proteins of Sin Nombre virus (SNV) and Andes virus (ANDV), their characterization by different immunoassays and comparison with the previously generated mAbs raised against a segment of PUUV N protein inserted into VLPs. All mAbs reacted strongly in the ELISA and Western blot test with the antigens used for immunization and cross-reacted to varying extents with N proteins of other hantaviruses. All mAbs raised against a segment of PUUV N protein presented on chimeric VLPs and both mAbs raised against the full-length AND/SNV N protein reacted with Vero cells infected with different hantaviruses. The reactivity of mAbs with native viral nucleocapsids was also confirmed by their reactivity in immunohistochemistry assays with kidney tissue specimens from experimentally SNV-infected rodents and human heart tissue specimens from hantavirus cardiopulmonary syndrome patients. Therefore the described mAbs represent useful tools for the immunodetection of hantavirus infection.

Biotechnological approaches to improve plant cold tolerance

This project was supported by the Lithuanian State Science and Studies Foundation grant N-07014. Project participants: Institute of Biotechnology, Institute of Horticulture and University of Agriculture.

An economical value and competitiveness of the important and industrial energy crops rape (Brassica napus), Miscanthus and horticultural plants as a source of colorants, medical and food compounds and their rootstocks is reduced by limited adaptation – winter hardiness and cold adaptation in Lithuania. The aim of this project was combine an experience of the participants in research on industrially important plants, gene engineering, and development of cold resistant plant selection technologies. The main goal of this integrated project was to develop transgenic plants with improved cold hardening and resistance properties. In addition, genetic transformation of the plants require prior investigation of traits implicated in cold hardiness that could be used to assess effects of interaction of transgenes with the endogenous mechanisms controlling acclimation processes.

The best known and probably the most cold-specific CBF/DREB cold resistance pathway was studied in Brassica napus. By homology-based PCR, analogs of cold inducible transcription factors CBF, ICE and MYB from B. napus were cloned. Transgenic lines of model plant A. thaliana have been constructed and were used in function analysis of BnCBF, BnICE and BnMYB in Arabidopsis. Proposed function of the BnICE protein as transcription factor was supported by the finding that it is localised in nucleus.

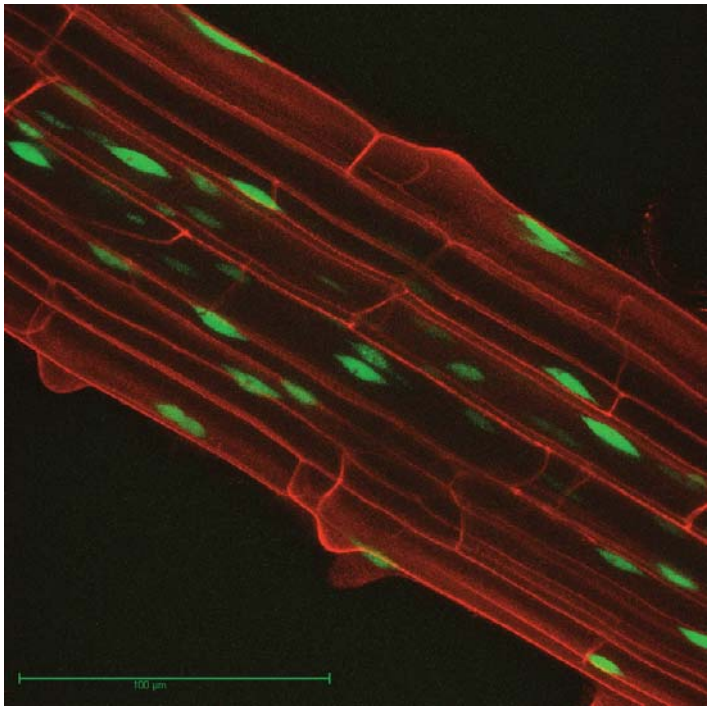

Figure 4. Expression of Bn-ICE-GFP in roots of transgenic A. thaliana 35S:BnICE-GFP plants. Green fluorescence - GFP, red fluorescence - propidium jodide stained cell walls.

Plant transformation vectors developed by IBT were used for genetic transformation of winter rape, Miscanthus and quince at Lithuanian institute of Horticulture and Lithuanian University of Agriculture. Further characterization of transgenic plants and cold resistance studies are ongoing.

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

The project was supported by the Lithuanian State Science and Studies Foundation grant N-07010 in 2007-2009.

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 were needed to evaluate diversity of and damage being done by phytoplasmas and viroids in valuable plants in Lithuania.

This project was aimed at identification of the harmful viroid and phytoplasma species in Lithuania, evaluation of their disperse in horticultural crops used in industrial biotechnology, development of molecular pathogen detection methods by designing new primer groups, evaluation of possibilities of horizontal DNA transfer within the phytoplasmas' genome and revealing the possibilities of growing viroid- and phytoplasma-free plants by applying thermo-, cryo-, and chemo-therapy in vitro.

In 2007-2009, the gardens, ecological plantations, greenhouses and a number of agricultural areas (potato plantations) were inspected in various regions of Lithuania. The samples from selected plants were collected and examined by applying Return Polyacrylamide Gel Electrophoresis, RT-PCR or RNA hybridization techniques to detect viroid RNAs and PCR assay to detect phytoplasmas. Over the three years of running this project, broadly world-wide detected viroids (e.g. PBCVd in pear trees and HSVd in one plum tree) were found in Lithuania as well. Despite a small part of all tested plants were viroid-infected, their detection and elimination from plants remain very important to maintain high productivity of plants grown for biotechnology industry.

Phytoplasmal infections were detected in a number of woody and herbaceous plants. New phytoplasma subgroup 16SrIII-T (first time in the world) and new cherry proliferation strain in 16SrI-B phytoplasma subgroup were detected in sweet and sour cherries in Lithuania. Phytoplasma detected in raspberries was classified in subgroup 16SrV-E (RuS strain) that was detected for first time in Lithuania. By using designed RCS-based primers it will become possible to detect phytoplasmal infection in a higher number of plant samples in shorter time. Acholeplasmas in tested plants were not detected.

It was found that the regime of thermotherapy should be chosen by plant's group. Application of thermotherapy under high temperature conditions for potato in vitro shoots was not purposeful as 98,0% of shoots did not survive. Shoots of woody plants formed new shoots after applying high or contrasting temperature conditions, however, their survival percentage and replication coefficient were lower than applying only low positive temperature. The results of chemotherapy experiments showed that both shoot survival percentage and shoot replication coefficient and their rooting capacity varied and depended on plant's genotype and tetracycline concentration. When running this project, proliferating in vitro cultures were obtained both from woody and herbal phytoplasma- and viroid-infected plants. Therapy systems were applied that enabled us to obtain infection-free microplants and to develop conditions that allow obtaining uninfected microplants during the shortest period of time in sufficiently high quantities. This factor is very important when working with industry valuable cultures.

Small Molecule Inhibitors of the Trimeric Influenza Virus Polymerase Complex

Collaborative project Fluinhibit (FP7 Health-2007-2.3.3.7: Supporting highly innovative inter-disciplinary research on influenza, grant No. 201634) was aimed to discover the inhibitors of RNA polymerase from influenza and prepare new platform for development of antiviral drugs based on polymerase inhibition. Availability of suitable amounts of viral polymerase was the basic requirement of the project. These tasks were successfully reached by IBT team and let us supply our partners of the project with the material for inhibitor selection and complex investigation.

For the expression in P. pastoris cassettes AOX-PA_AOX-Strep-PB1 and AOX-PA_AOX-PB1-Strep were individually transferred into the vector pPIC3.5K-PB2-6His (behind AOX-PB2-6His-TT cassette). Two variants of expression vectors were constructed for simultaneous expression of recombinant genes coding polymerase subunits: PA, Strep-PB1 or PB1-Strep and PB2-6His. P. pastoris cells were transformed with final expression vectors, selected on media with G418 and induced with methanol. The synthesis polymerase subunits have been proved by Western blot.

Influenza A RNA polymerase was successfully overexpressed in S. cerevisiae using two yeast expression systems: 1) all three Flu A RNA polymerase subunits (PA, PB1 his tagged at the N-end and cap binding mutant of PB2 Strep tagged at the C-end) coding genes were cloned into the same expression vector pFGG3 under the control of galactose inducible promoters; 2) the system was designed so that PA and PB1 subunits were cloned into one expression vector (pEX1) and PB2 into another one (pEX2). Both systems proved to be successful. The expression of all three Flu A RNA polymerase subunits showed to be more effective using two-plasmid based yeast expression system. It was possible to purify three subunits using his-tag attached to the N-end of PB1 subunit. RNA polymerase subunits: PA, PB1 and PB2, each separately, were successfully expressed in yeast Sacharomyces cerevisiae and Pichia pastoris cells GS115. For every subunit the suitable tag type and its location were selected considering their efficiency of expression, fitness for purification and further applications. The efficiency of expression of all polymerase subunits were tested using two different yeast expression vectors (pFGG3 and pFGAL7) in three different yeast S. cerevisiae strains (FH4 wild type (wt), AH22 and 214 Δpep4). In order to express PA/PB1 dimer in S. cerevisiae, both subunits coding genes were cloned in to the same expression vector pFGG3 under the control of galactose inducible promoters. For the expression in yeast several constructions were used and tested for influenza A virus RNA polymerase PA/PB1 dimer expression in S. cerevisiae 214 Δpep4 strain. The most efficient production of PA/PB1 dimer was achieved in case when PA without tag was expressed from promoter (GAL-10) and the gene of PB1 tagged at the N-end was expressed under control of GAL-7, which is more potent than GAL-10. Yeast expressed PA/PB1 dimer was purified using procedure of three steps: precipitation by ammonium sulfate, then purification by metal-affinity chromatography and by gel filtration. Using optimized purification protocol it was possible to achieve more than 90% purity of yeast expressed PA/PB1 dimer. Yeast expressed PA/PB1 dimer has been purified and provided to project partners for further applications. Overall success of the Fluinhibit project let us to extent our investigation in the new FP7 collaborative project called Flucure (FP7-Influenza-2010, grant No. 259972) which was highly ranked during the selection and will start from October 2010 ending in 2014.

International Collaboration
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Contracts
Microimmune Ltd., London, U.K.
Micromun GmbH, Germany
Friederich-Loeffter-Institut, Germany
Euroimmune AG, Germany
Measles Research Centre, China
UAB Profarma, Lithuania
UAB Fermentas, Lithuania
DiaSorin S.p.A.
Abcam Ltd., U.K.

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21. Pumpens P., Ulrich R.G., **Sasnauskas K.,** Kazaks A., Ose V., Grens E. Construction of novel vaccines on the basis of virus-like particles. Hepatitis B virus proteins as vaccine carriers. Khudyakov YE, editor. Medicinal protein engineering. 2008. Boca Raton, CRC Press LLC, p.p. 205-248.
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23. Žvirblienė A., Kučinskaitė-Kodzė I.. **Plečkaitytė M., Žvirblis G.**. Monokloniniai antikūnai prieš vaginoliziną (Monoclonal antibodies against vaginolysin), Patent No. 2009 012 (LT).
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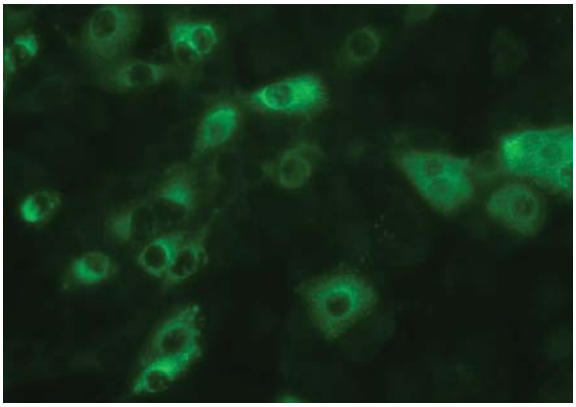
The Laboratory of Immunology and Cell Biology consists of three research groups. In 2008-2010, the research was focussed to the following topics: antigenic characterization of recombinant viral and bacterial proteins, development of monoclonal antibodies (Dr. A.Žvirblienė), regulation of gene expression by alternative splicing (Dr. A.Kanopka), molecular epidemiology of tuberculosis (Dr. P.Stakėnas).

Antigenicity studies of recombinant viral proteins

This work 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 nucleocapsid (N) proteins of paramyxoviruses, such as human parainfluenza virus type 3 (hPIV3) and Menangle virus has been investigated. Recombinant N proteins were expressed at the Laboratory of Eukaryote Gene Engineering.

The yeast-expressed N proteins used in this study were self-assembled into nucleocapsid-like structures (NLPs) similar to that of native virus. To identify B-cell epitopes, we have employed monoclonal antibodies (MAbs) raised against recombinant N proteins as well as human sera from virus-infected individuals. The localization of B-cell epitopes was studied using recombinant overlapping N protein fragments, PepScan analysis and competitive ELISAs. The majority of MAb epitopes were mapped within the C-termini of N proteins. Cross-inhibition studies with human sera demonstrated similar localization of B cell epitopes recognized by serum antibodies from naturally infected individuals, which revealed a clear antigenic similarity between recombinant and virus-derived N proteins. Our data suggest that different paramyxoviruses may contain highly conserved surface-exposed structures within the C-termini of N proteins that are well accessible to B cells and elicit antibody response. These findings may have important implications for the design of new vaccines and diagnostic reagents (Zvirbliene et al., Viral Immunol. 2009, Zvirbliene et al., Arch Virol. 2010).

A



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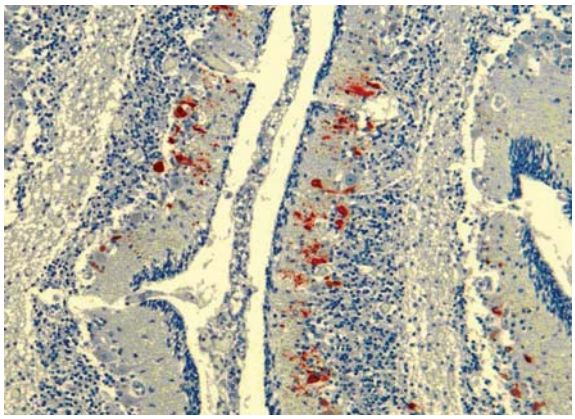

Figure 1. Immunochemical detection of virus-infected cells using monoclonal antibodies raised against recombinant N proteins of hPIV3 (A) and Menangle virus (B).

Functional and immunological characterization of recombinant vaginolysin

Vaginolysin (VLY) is the main virulence factor released by Gardnerella vaginalis. VLY is a protein toxin that acts as a hemolysin. It belongs to the group of cholesterol-dependent cytolysins (CDCs), a large family of related pore-forming toxins found in five different genera of Gram-positive bacteria. Generation of MAbs against bacterial cytolysins may promote elucidation of the relationship between their structure and mode of action.

In the current study, we have developed and characterized a panel of MAbs against recombinant VLY and demonstrated that several MAbs neutralize VLY in the in vitro hemolytic assay. By using a series of recombinant proteins we have studied the specificity of the MAbs and mapped the epitope recognized by the most potent neutralizing MAb. The epitope for this MAb was localized near the N-terminus of VLY and included the conserved motif (VAARMQYD, aa 189-196) supposed to be involved in VLY oligomerization. Furthermore, we have employed the MAbs in a sandwich ELISA for VLY quantitation. In conclusion, the MAbs described in the current study may be useful for structural and functional studies of VLY as well as immunodetection of VLY in biological specimens (Zvirbliene et al., Toxicon 2010, PCT/LT2009/000005).

This work was supported by the Lithuanian State Science and Studies Foundation/Lithuanian Science Council (grant No. N-17)

Dr. M.Plečkaitytė and Master student J.Alesiūtė investigate immunochemical properties of vaginolysin

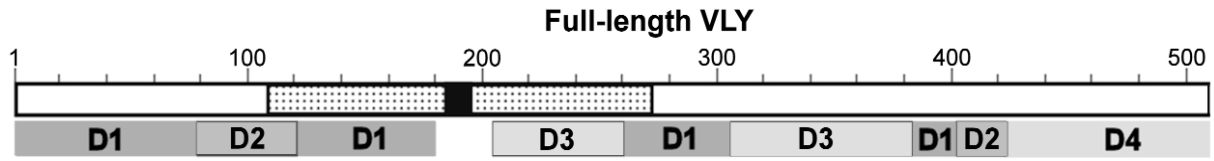

Figure 2. Schematic representation of VLY domains (D1-D4). Dashed area indicates VLY region recognized by the neutralizing monoclonal antibody.

Master student V.Žilaitytė, Dr. A.Žvirbliene, junior scientist I.Šežaitė, PhD student I.Kučinskaitė-Kodzė, junior scientist R.Lasickiene develop new hybridoma cell lines

Junior scientist E.Jakubauskienė and PhD student I.Pečiulienė investigate HIF-3a expression

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

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 S.cerevisiae. These chimeric HaPyV-VP1 VLPs have been shown to induce a strong antibody response against the inserts (Gedvilaite et al., 2004, Zvirbliene et al., 2006). Chimeric VLPs efficiently 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, such as tumor-associated epitopes (Dorn et al., Viral Immunol, 2008). Moreover, it was demonstrated that the insert-specific antibodies 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 highly efficient immunogens for hybridoma technology and provide a promising alternative to chemical coupling of synthetic peptides to carrier proteins.

This work was supported by UAB Fermentas (Contract No. 1224).

Regulation of hypoxia-inducible factor HIF-3α 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.

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. 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.

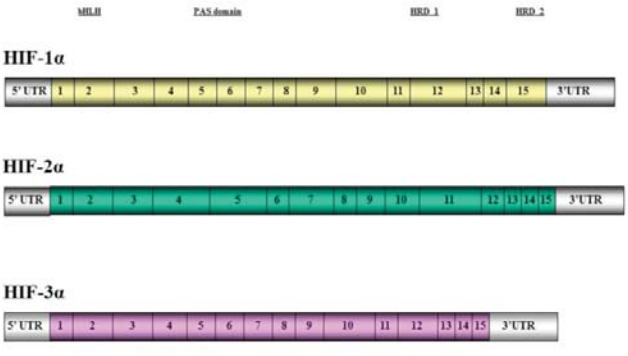

Figure 3. Schematic representation of HIFα subunit mRNA variants detected in the cell.

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. Biological responses to hypoxia involve induction of transcription of a network of target genes, a process which is coordinately regulated by hypoxia-inducible transcription factors (HIFs). three structurally related bHLH transcription factors (HIF-1, HIF-2 and HIF-3). HIFs recognize hypoxia response elements of targets genes as hetrodimeric complexes (HIF-1α, HIF-2α and HIF-3α) with the transcription factor Arnt.

A splice variant of HIF-3, inhibitory PAS domain protein (IPAS), inhibits the dimerization of HIF-1 and ARNT. IPAS protein contains a bHLH domain and a PAS domain, which are the common structures present in the HIF family. 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 are involved in dependable from oxygen tension pre-mRNA splicing regulation. Changing cellular expression of these factors or totally inhibiting their expressions should lead cancer cells to apoptosis. Identification splicing factor expression role in cancer cells might reprogram cellular events and could 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 program (project Euroxy) and Framework 7 program (project Metoxia).

Molecular epidemiology of Mycobacterium tuberculosis

This work was performed in collaboration with National Tuberculosis and Infectious Diseases University Hospital (Vilnius, Lithuania).

Tuberculosis 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 tuberculosis cases and 1.8 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. Multidrug-resistant tuberculosis is treated by using the second-line antituberculosis drugs. 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 2008, there were 2097 tuberculosis cases (notification rate 62.45 per 100 000 of population), spread of MDR TB was one of the highest in the world (13.17% of the tuberculosis 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 have continued characterisation in detail population of M. tuberculosis including genetic determinants of drug resistance which has circulated in Lithuania.

Almost nine hundred M. tuberculosis clinical isolates, including 30% MDR strains, from different patients were examined by restriction fragment length polymorphism typing using the insertion element IS6110 as a probe (IS6110-RFLP). A phylogenetic structure of the searched population was defined by spoligotyping Overall, analysis of genotypes demonstrated that epidemiological situation of tuberculosis does not change essentially. Stabilization of situation continues, but the break of most dangerous transmission chains does not occur.

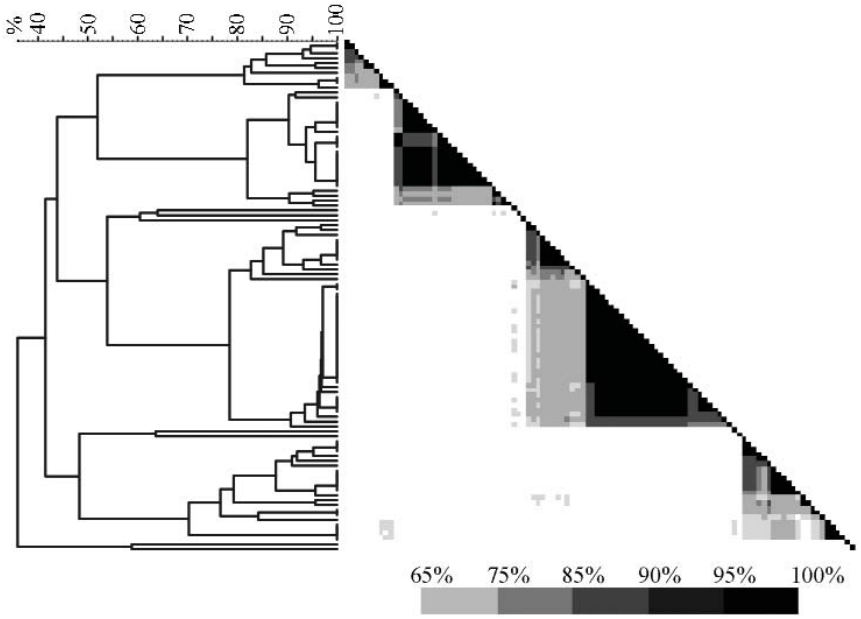

Figure 4. Dendrogram and similarity matrix of IS6110-RFLP patterns showing relatedness of multidrug-resistant M.tuberculosis strains circulating in Lithuania

Until recently, IS6110-RFLP and spolygotyping were recognized as the reference methods. Currently, IS6110-RFLP assay are replacing by 24-locus MIRU-VNTR typing as the gold standard for genotyping of M. tuberculosis. Therefore, in the frame of the TB PAN-NET project we have started molecular epidemiology study of drug resistant strains in Vilnius by using MIRU-VNTR typing as well. The results demonstrated a good correlation between these both techniques.

Investigation of mutations conferring M. tuberculosis resistance to the second-line drugs was continued by search for mutations associated with resistance to ofloxacin and reserve aminoglycosides. Currently, our collections consist of 126 ofloxacin-resistant isolates and 152 kanamycin-resistant, including 26% amikacin-resistant isolates, from different patients and characterized in detail by the means of genotyping. However, only 82% of ofloxacin- resistant isolates carried resistance conferring mutations in the quinolone resistance-determining regions (QRDR) of gyrA or gyrB genes and 16% of kanamycin-resistant strains had mutations in the rrs gene. These results demonstrated that significant part of resistant cases cannot be explained by the presence of mutations in the well-recognized hot spot regions of M. tuberculosis chromosome (Devaux et al., Euro Surveill. 2010). Search for novel mutations associated with M. tuberculosis resistance to these drugs is in progress.

This work was supported by the Lithuanian State Science and Studies Foundation (grant No. C-01), EC FP7 Health Programme (Project TB PAN-NET, grant No. 223681), and Agency for Science, Innovation and Technology (grants No. 31V-169, No. 31V-32).

Dr. P.Stakėnas and junior scientist D.Bakonytė perform genotyping of M.tuberculosis

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Contracts

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Santa Cruz Biotechnology, USA

Publications 2008-2010

Journal Articles

1. **Zvirbliene A, Pleckaityte M, Lasickiene R, Kucinskaite-Kodze I,** Zvirblis G. Production and characterization of monoclonal antibodies against vaginolysin: Mapping of a region critical for its cytolytic activity. Toxicon. 2010, 56:19-28.
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PATENT APPLICATION

10. *Zvirbliene A., Kucinskaite I., Pleckaityte M.,* Zvirblis G. Monoclonal antibodies against vaginolysin. PCT/LT2009/000005

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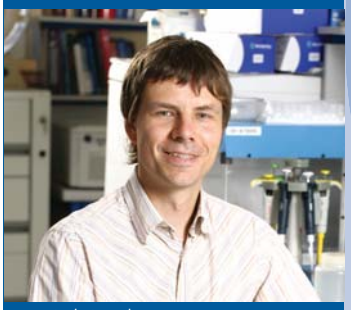

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Associated Scientists on leave
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The Laboratory of Biothermodynamics and Drug Design (LBDD) was founded in 2006 in the place of the former Laboratory of Recombinant Proteins. The LBDD designs novel chemical compounds with anticancer activity. The efficiency of both naturally occurring and synthetic compounds is evaluated by structural biothermodynamics and molecular modelling methods.

The laboratory's personnel consist of five teams according to their research activities:

The Team of Molecular and Cell Biology, headed by Dr. Jurgita Matulienė (Ph. D. in cell biology from the University of Minnesota, USA, 2003) produces drug target proteins by gene cloning, expression in *E.coli*, insect, or mammalian cells, and chromatografic purification of large quantities of active proteins sufficient for biothermodynamic measurements of binding chemical compounds. Several projects involve the design of protein domain constructs. Live human cancer cells are cultured for the evaluation of compound anticancer activity.

The Team of Organic Synthesis, headed by Dr. Virginija Dudutienė (Ph. D. in organic synthesis from the Vilnius University, 2005) synthesizes compounds that are designed to bind drug target proteins. Compounds are designed by computer docking, molecular modelling, and comparison with naturally occurring or previously synthesized compound functional groups. The special interest and capabilities of the group are in the field of synthesis of compounds with multiple conjugated aromatic heterocycles.

The Team of Biophysics, headed by Dr. Daumantas Matulis (Ph. D. in biochemistry, molecular biology and biophysics from the University of Minnesota, USA, 1998) measures compound/ligand binding to target proteins by isothermal titration calorimetry (ITC), thermal shift assay (ThermoFluor®), and pressure shift assay (PSA). The team performs the characterization of protein stability in the presence of various excipients and the measurement of enzymatic activity.

The Team of Computer Modelling is responsible for the *in silico* docking of large compound libraries and the analysis of X-ray crystal structures of synthetic compound – protein complexes solved in collaboration with Dr. Saulius Gražulis group in the Laboratory of Protein – DNA interactions. Molecular modelling of candidate compounds often predicts novel compounds with improved binding capabilities. The group, together with several collaborating scientists 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 Team of Applied Biocatalysis, headed by Inga Matijošytė (Ph. D. in biochemistry and biocatalysis from Delft University, The Netherlands, 2008), established in 2007 in conjunction with the start of the Program on the Development of Industrial Biotechnology in Lithuania, applies enzymes as biocatalysts in organic synthesis to achieve desired conversions. Team's research is directed towards the search for enzymes with new functionalities and their development towards applied biocatalysts.

Research Projects

Several protein targets have been selected for the investigation of protein – ligand binding thermodynamics and the design of novel ligands with desired properties. A family of human carbonic anhydrases, heat shock proteins, and several signal-tranducing proteins were chosen as anticancer drug targets.

Novel methods and thermodynamic approaches are being used and developed in the laboratory. Detailed thermodynamic description of natural compound – protein interaction provides clues to improved ligand affinity and specificity. In addition to the Gibbs free energy, enthalpy, entropy, and the heat capacity, the laboratory studies the volume and compressibility of the protein – ligand interactions.

The laboratory has been recently invited to write a review chapter on the thermal shift assay in Royal Society of Chemistry Biomolecular Sciences No.22 book (Cimmperman and Matulis, 2011).

Carbonic anhydrases as anticancer drug targets

Carbonic anhydrases (CAs), a group of 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 antiobesity, anticancer, and anti-infective drugs (Supuran, 2007). CAs catalyse a simple reaction – the conversion of CO_2 to the bicarbonate ion and protons. There are 12 catalytically active CA isoenzymes in humans. A number of CA inhibitors, mostly unsubstituted sulfonamides, have already been designed. However, most present inhibitors are insufficiently selective for target CA isozymes, such as hCAIX and hCAXII, anticancer targets.

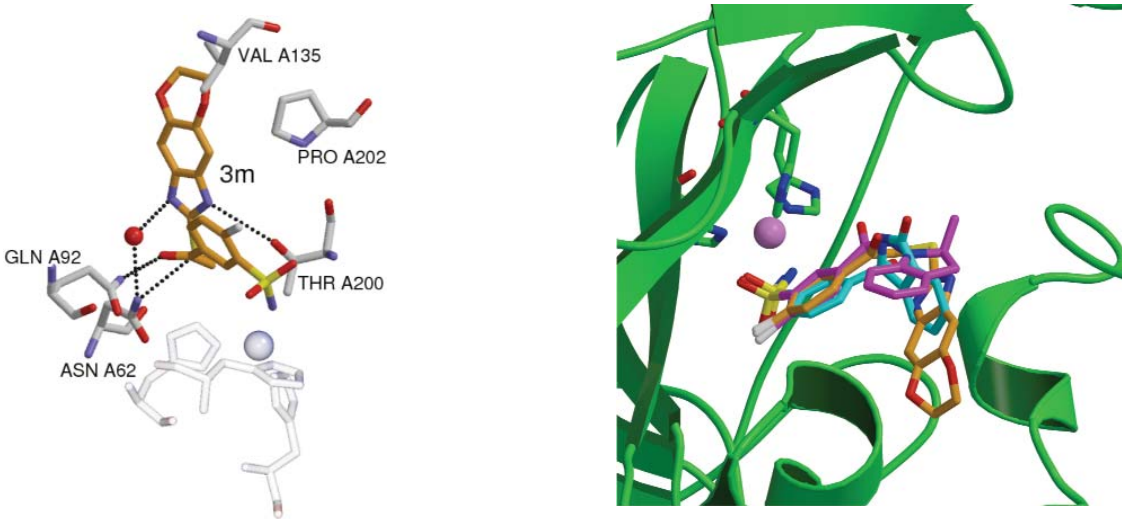

Figure 1. Left panel. View of compound **3m** bound in the active center of hCA II. The Zn atom, His94, His96, and His119 are shown as transparent, inhibitors are shown in orange. Right panel. Superposition of the hCA II–indapamide (magenta), hCA II-chlorthalidone (cyan) with hCA II – **3m** (orange). Zinc ion is shown as pink sphere, His94, His96, His119, and protein secondary structures are in green. The crystal structure was solved in collabora-tion with dr. Saulius Gražulis group, Laboratory of Protein-DNA Interactions (Capkauskaite et al, 2010).

Here at the LBDD we have cloned and purified most soluble CAs and truncated versions of CAs with removed transmembrane domains. Laboratory participated in the characterization of hCA IX (Hilvo et al, 2008). The organic synthesis team together with collaborators designed and synthesized over 200 novel compounds that bind CAs with submicromolar affinity. Several novel groups of CA inhibitors exibited high affinity and appreciable selectivity towards selected CA isozymes (Dudutiene et al. 2007; Baranauskiene et al. 2010; Sudzius et al. 2010; Capkauskaite et al. 2010).

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.

Our laboratory is interested in the mechanism of Hsp90 action and the thermodynamics of inhibitor binding. Thermodynamics of a natural compound radicicol binding to human Hsp90 alpha and beta isozymes and yeast Hsc82 was studied by isothermal titration calorimetry, thermal shift assay, and the pressure shift assay. These studies provided an unusual and detailed picture of Hsp90 inhibitor binding energetics. Radicicol bound Hsp90 with exceptionally large

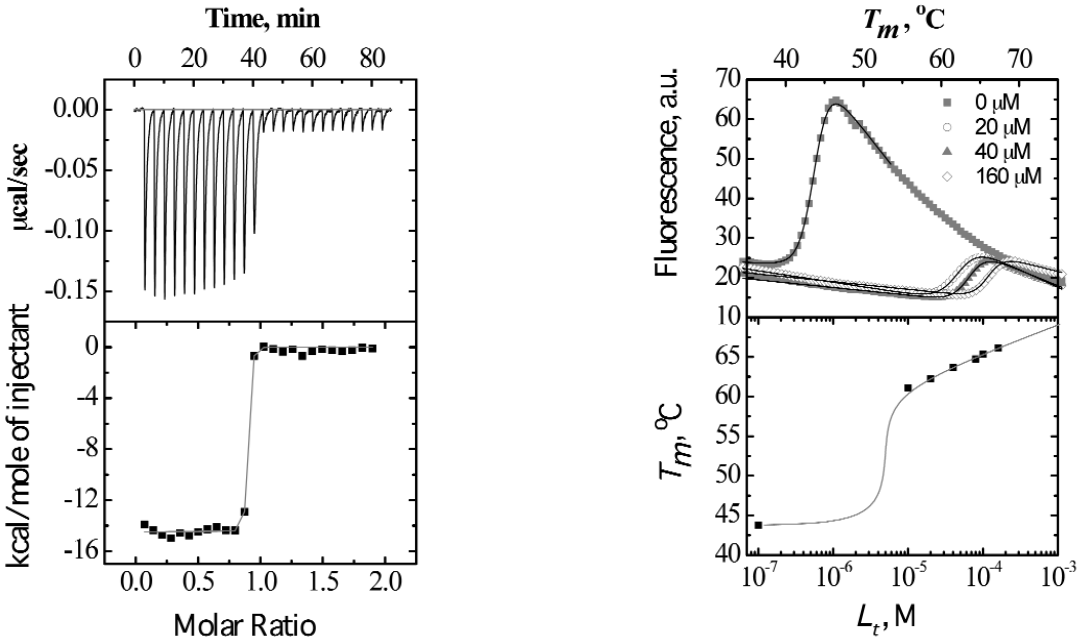

Figure 2. Radicicol binds Hsp90 with high affinity in the order of 100 pM K_d. Titration calorimetry (graphs on the left) yield a stepwise curve – too steep to determine the Kd. Thermal shift assay (graphs on the right, upper panel – fluorescence dependence on temperature at various added ligand concentrations, lower panel – T_m dependence on added ligand concentration) provides quite precise measurement of the binding constant.

exothermic enthalpy and volume of binding (Zubriene et al 2009, Zubriene et al. 2010, Toleikis et al, submitted).

A novel group of inhibitors has been designed and synthesized based on similarity to radicicol and several inhibitors designed by Vernalis, UK. Our series of inhibitors were significantly easier to synthesize and possessed comparable activity towards HeLa and osteosarcoma cells (Cikotiene et al, 2009). Structure – thermodynamics analysis provided further insight into the mechanism of such inhibitor binding to Hsp90.

Ligand binding equilibria by protein high pressure denaturation

The volume changes accompanying ligand binding to proteins are thermodynamically important and could be used in the design of compounds with specific binding properties. Measuring the volumetric properties could yield as much information, as the enthalpic properties of binding. Pressure-based methods are significantly more laborious than temperature methods and are underused. The pressure shift assay (PressureFluor, analogous to the ThermoFluor, thermal shift assay) uses high pressure to denature proteins. The PressureFluor method was used to study the ligand binding thermodynamics of Hsp90. Ligands stabilize the protein against pressure denaturation, similar to the stabilization against temperature denaturation.

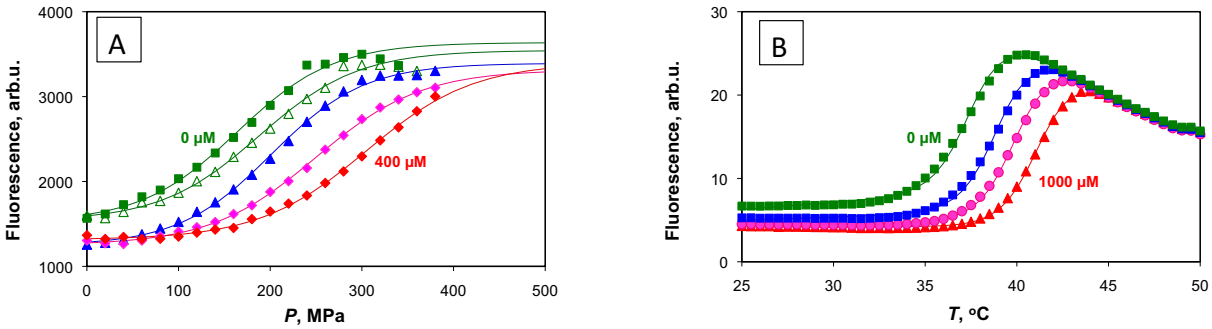

Figure 3. Pressure shift assay (panel A) and thermal shift assay (panel B). Elevated pressure or temperature causes protein denaturation. Addition of a ligand of an increasing concentrations increases protein melting pressure and melting temperature.

Applied biocatalysis projects

The limited number of suitable and well characterized biocatalysts delays the progress in the application of enzymes in the synthesis of compounds for materials, pharmaceuticals and chemicals. The Team of Applied Biocatalysis seeks to identify biocatalysts with novel activities by the three most common ways: screening for enzymes (environmental samples, enzyme and strain collections, expression databases), development of biocatalyst (directed evolution, genetic engineering, development of analytical systems) and the application of biocatalysts (immobilization, recycling, proof of principle, activity/selectivity, stability, and reaction media).

The research focuses on biocatalytic systems employing lipolytic, hydrolytic, proteolytic and oxidative enzymes. We strive to meet scientific challenges in combination with application-oriented research.

Services

The LBDD is seeking to license out the compounds described in patent applications. The LBDD is also interested in the collaborations where our expertise in the determination of compound – protein binding thermodynamics and recombinant protein stability characterization could be applied. Protein – ligand binding constants and protein thermal stability profiles at hundreds of conditions may be determined in a single experiment by consuming microgram quantities of protein.

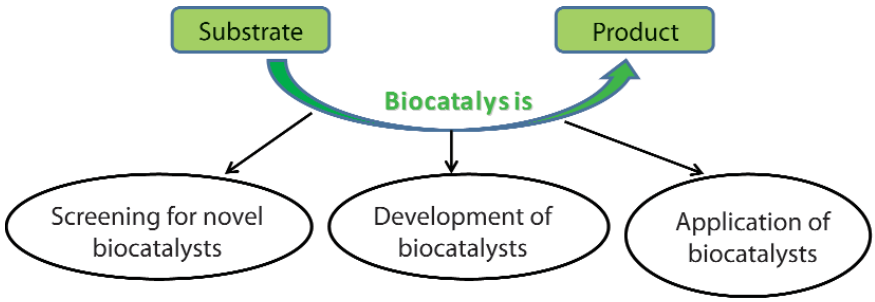

Figure 4. Reseach areas of the Team of Applied Biocatalysis

CONFERENCES AND COLLABORATION

The LBDD regularly participates in many international conferences and symposiums, including:
International Conference on the Hsp90 chaperone machine.
International Conference on the Carbonic Anhydrases.
International Conference on High Pressure Bioscience and Biotechnology.
Biothermodynamics Symposium.
European Biophysics Congress.
Biophysical Society Annual Meeting.
Gibbs Conference on Biothermodynamics.
International Conference of Lithuanian Biochemical Society.

The LBDD has ongoing collaborations with a number of research laboratories and industry worldwide, including:
Institute of Medical Technology, University of Tampere, Finland.
University of Florence, Italy.
International Institute of Molecular and Cell Biology, Warsaw, Poland.
Pharmaceutical Research and Development, L.L.C., Johnson&Johnson, USA.
Centre for Structural Biochemistry, Montpellier, France.
Institute of Organic Synthesis, Riga, Latvia.
Institute of Organic Chemistry, University of Tubingen, Germany.
Chemistry Centre of Madeira, University of Madeira, Portugal.
Cancer Research Centre, University of Edinburgh, UK.
University of Bristol, UK.
Delft University of Technology, Netherlands.
Faculty of Chemistry, Vilnius University, Lithuania.
Faculty of Natural Sciences, Vilnius University, Lithuania.
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Lithuanian University of Agriculture, Kaunas, Lithuania.
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UAB „BIOK", Vilnius, Lithuania.
UAB „Biocentras", Vilnius, Lithuania.
Nature Research Centre, Institute of Botany, Vilnius, Lithuania.

GRANTS

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EC FP6 Marie Curie International Reintegration (J. Matulienė)
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*recommended by Faculty of 1000 Biology

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In the post-genomics era computational methods are 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. Computational biology and bioinformatics are helping to make sense of all this vast biological data by providing tools for performing large-scale studies. In addition, computational biologists are utilizing available experimental data to improve various analytical and predictive methods that could help address specific biological problems.

Research carried out in our laboratory covers a broad range of topics that together can be described as Computational Studies of Protein Structure, Function and Evolution. There are two main research directions:

- Development of methods for the detection of protein homology from sequence data, comparative modeling and analysis of protein three-dimensional structure.
- Application of computational methods for structural/functional characterization of proteins and their complexes; design of novel proteins and mutants with desired properties. Our main focus are DNA-interacting proteins, in particular those functioning in DNA replication, repair and recombination.

Development of methods for the detection of protein homology from sequence data, comparative modeling and analysis of protein three-dimensional structure

Profile-based detection of remote evolutionary relationships between protein families

Related (homologous) proteins tend to have similar three-dimensional (3D) shapes and similar molecular functions. Therefore, the detection of evolutionary relationship serves as a powerful technique for the annotation of uncharacterized proteins and also as a first step in comparative modeling of protein 3D structure.

When the relationship between proteins is very distant, the sensitivity of popular sequence comparison methods such as BLAST or even PSI-BLAST may be not sufficient to detect it. In such cases methods based on comparison of protein multiple sequence alignments, represented either as numerical sequence profiles or as Hidden Markov Models (HMMs) at present are among the most effective. However, even those methods fail to identify many of the remote evolutionary links, underscoring the need for increased sensitivity.

Aiming at improvement of remote homology detection, we have developed a new profile-based method, COMA (Comparison Of Multiple Alignments). COMA works by performing pairwise comparison of sequence profiles constructed from the multiple sequence alignments. The two features that distinguish COMA from other profile-profile comparison methods most are position-dependent gap penalties and the estimation of significance of the compared profile pair. Position-dependent gap penalties provide biologically more relevant treatment of insertions and deletions in profile alignments, while the introduction of a global scoring system leads to a rigorous statistical assessment of profile relatedness. We benchmarked our method together with other top-of-the-line profile-based methods (HHsearch, COMPASS and PSI-BLAST). Evaluation results showed that at the level of protein domains our method compares favorably to all other tested methods. In the course of the assessment we even found cases when our new method outperformed DALI, which works by comparison of known 3D protein structures (Fig. 1). These results further suggest that even if the 3D structure is known, sequence-profile based methods may reveal additional hidden evolutionary links with other proteins.

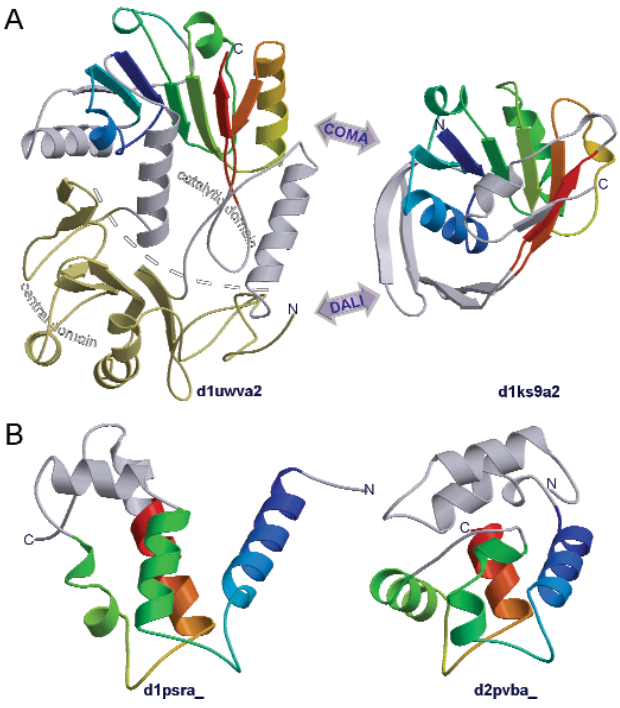

Figure 1. Examples of COMA performing better than DALI, a structure-based method. (A) COMA but not DALI correctly aligns d1ks9a2 with the related catalytic domain of d1uwva2. Coloring of d1ks9a2 and the catalytic domain of d1uwva2 corresponds to the progression of polypeptide chain from N- (blue) to C-terminus (red). (B) Homologous pair of EF-hand proteins, human psoriasin (d1psra_) and pike parvalbumin (d2pvba_) is identified by both COMA and DALI, but the alignment by COMA is more accurate.

COMA has been implemented as a standalone software package, making it suitable for large–scale studies. However, the use of standalone tools requires a certain level of expertise and substantial computer resources. Therefore, to make this method accessible for larger biological community, we have also developed a freely accessible web server (http://www.ibt.lt/bioinformatics/software/coma/). The server features a simple and intuitive user interface for setting up homology search jobs. The homology search is done by comparing query-based sequence profile against the selected profile database (at present SCOP, PDB and PFAM). The server output gives a concise information regarding structure, function and other relevant information for the reported hits. Hits are linked to original databases (PDB, SCOP or PFAM) and to the search results within the collection of PubMed articles using annotation of hits as keywords. If the search was performed against PDB or SCOP, the server provides a possibility to generate a 3D model of the query sequence based on the corresponding alignment.

We believe that the COMA method will be useful for researchers in uncovering interesting novel links in the proteome universe.

Identification of new homologs of PD-(D/E)XK nucleases by combination of Support Vector Machines and sequence profile-profile alignments

PD-(D/E)XK nucleases comprise a large and extremely diverse group of proteins that are involved in various processes of nucleic acid metabolism. As it happens, the name of this large group of nucleases derives from the highly conserved 'PD' (in many cases only 'D') and '(D/E)XK' ('X' denotes the non-conserved position) active site motifs. Typically, different PD-(D/E)XK families share little or no recognizable sequence similarity. Although they all have a common structurally conserved core, the latter is often elaborated with large non-conserved peripheral elements. This makes it hard to recognize the similarity between PD-(D/E)XK domains not only by sequence comparison but sometimes even by structure comparison. Therefore, we considered to develop a new method specifically tailored for the detection of new PD (D/E)XK homologs. To make use of all the available structural and functional information of PD (D/E)XK domains, we developed a method that uses combination of a supervised machine learning method, Support Vector Machines (SVMs) and alignments obtained with HHsearch, a sensitive profile-profile homology search method. Using our newly developed method we were able to detect several new families in the PFAM database and assign a number of restriction endonucleases to the PD-(D/E)XK type. To make the method accessible for broader biological community we implemented it as a user-friendly web server accessible at: http://www.ibt.lt/bioinformatics/software/pdexk/.

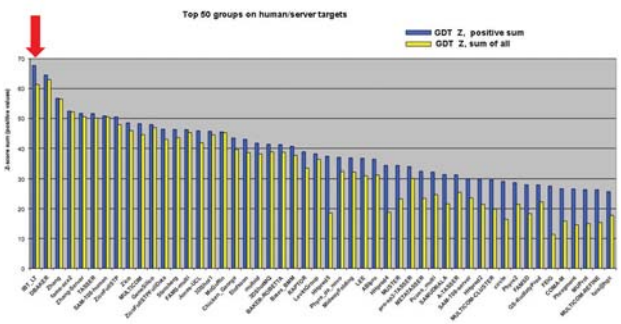

Figure 2. CASP8 protein structure prediction results for the top 50 expert groups. Results of our group (IBT_LT) are indicated with a red arrow.

Development and testing of comparative (template-based) modeling methods

Over the years we have been continuously working on the improvement of comparative (template-based) modeling methodology. In computational biology it is important to do regular "reality checks" to find out where your methods stand in relation to others. Additional motivation was to test our new homology detection method, COMA. Therefore, in 2008 we took part in a world-wide experiment on protein structure prediction known as CASP (Critical Assessment of Structure Prediction). Our team, named IBT_LT (Institute of Biotechnology, Lithuania), together with over 150 other predictor groups around the world tried to compute the closest possible 3D shapes of more than 50 proteins provided by the organizers.

IBT_LT team at the CASP8 meeting in Sardinia

The structures of these proteins had been unknown at the time of the experiment and were released to the public only after predictions were collected. This setting ensured that the testing of protein structure prediction methods was done in the most objective way. After the results of the CASP8 experiment were assessed by independent experts, it turned out that the results obtained by our team (IBT_LT) were ranked first (Fig. 3) in the most representative, template-based, protein structure prediction category (see also Cozzetto et al. (2009) Proteins: 77 Suppl 9:18-28). The detailed comparison of the performance by different groups in the CASP8 experiment is available at the UC Davis Prediction Center website (http://predictioncenter.org/casp8/groups_analysis.cgi). Our methods used in CASP8 and the obtained results have been reported in the invited article published as part of the special issue of Proteins: Structure, Function, and Bioinformatics [2].

Voroprot: an interactive software tool for visualization and analysis of various geometric features of protein structure

The ability to visualize and analyze geometric features of the 3D protein structure is fundamental in protein modeling, residue packing, docking, protein-protein interactions and other computational studies. Most interactive viewers are limited to standard representations of protein structure such as lines, cartoon-like diagrams, CPK representation and, sometimes, surfaces. To address the shortage of advanced protein structure analysis software, we developed Voroprot, a tool that extensively relies on the Voronoi and Apollonius diagrams and the Apollonius graph. Voroprot can construct interatomic contact and solvent accessible surfaces. It can also find internal cavities and pockets. In addition, Voroprot can construct a triangulated representation of the 3D protein structure, which is useful in investigation of protein surface curvature. Voroprot allows the visualization of every constructed geometric object and is capable of producing publication-quality images (Fig. 3). Voroprot is an easy setup standalone application available for Windows, Linux and Mac OS X platforms. Voroprot is free for academic use and can be downloaded from http://www.ibt.lt/bioinformatics/software/voroprot/.

All the methods that have been developed in our laboratory can be accessed through our website at: http://www.ibt.lt/bioinformatics/software/.

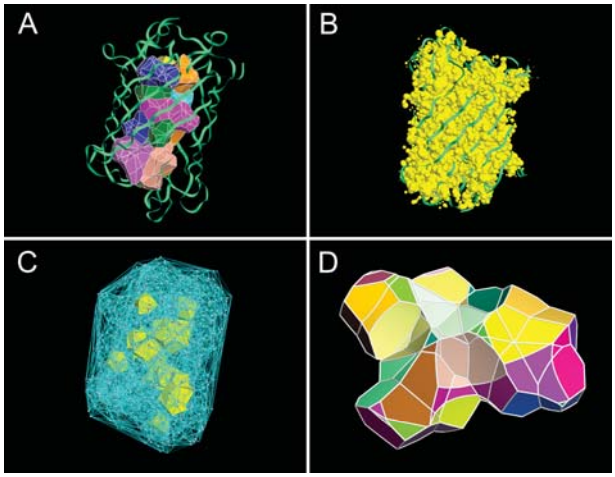

Figure 3. Visual analysis of the crystal structure of green fluorescent protein (GFP; PDB code: 1EMA) using Voroprot. (A) The Voronoi cells of the atoms of some of the residues inside. (B) Cavities inside the protein structure found by rolling water molecule on the protein surface. (C) The Apollonius graph of GFP atoms with some of the cavities shown in yellow. (D) The Voronoi cells of the chromophore atoms with their faces colored by identities of their neighbours.

Application of computational biology methods to specific biological problems

An important element of our laboratory research is projects in which computational methods alone or combined with experiments (in collaboration with experimental labs) are applied to address specific biological questions. Most of these ongoing projects involve proteins participating in DNA metabolism, in particular in DNA replication and repair. One of the projects listed below is described in detail.

- Computational analysis of the nature and distribution of DNA replication processivity components in genomes of double stranded DNA viruses
- Computational analysis of evolutionary distribution and structural properties of bacterial polymerase III catalytic subunits
- Computational identification and characterization of putative Type I and III restriction-modification systems in bacterial genomes.
- Elucidation of the three-dimensional structure and molecular mechanisms of the T4 bacteriophage replisome (collaboration with Prof. Virgis Šikšnys, Institute of Biotechnology)
- Computational/experimental studies of molecular functions of Elg1, a protein involved in the maintenance of genome stability in eukaryotes (collaboration with Prof. Martin Kupiec, Tel Aviv University)
- Molecular mechanisms of yeast Rad5 and its human ortholog, HLTF, in conferring DNA damage tolerance (collaboration with Dr. Lajos Haracska, Biological Research Center, Szeged)
- Molecular mechanisms of M. tuberculosis DNA mutagenesis (collaboration with Prof. Valerie Mizrahi, University of the Witwatersrand, Johannesburg)

Computational modeling in studies of mechanisms of M. tuberculosis DNA mutagenesis

The emergence of drug-resistant Mycobacterium tuberculosis (Mtb) strains is an important world-wide problem. The major cause of drug resistance in Mtb is thought to be the inducible mutagenesis. In Mtb, the induced base-substitution mutagenesis is dependent on DnaE2, the C-family DNA polymerase, and two other proteins, Rv3395c (ImuA') and Rv3394c (ImuB). Rv3395c is a protein of unknown function very distantly related to RecA (Fig. 4), and Rv3394c is its downstream partner predicted to encode a specialist Y-family DNA polymerase.

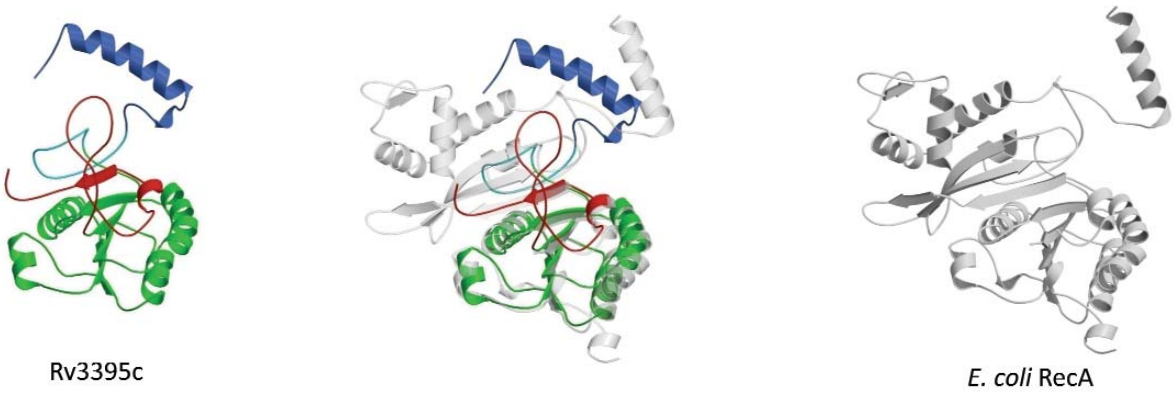

Figure 4. Comparison of a MTB ImuA' (Rv3395c) model with the RecA crystal structure. ImuA' appears to be a "crippled" RecA homolog. It lacks the C-terminal subdomain and ATP-binding site.

Previously, these genes were identified as putative mycobacterial components of an imuA'-imuB-dnaE2 mutagenic cassette widespread in bacteria. Using computational techniques we have generated models for all three Mtb proteins. Combining these models with sequence analysis we showed that although Rv3394c is related to Y-family DNA polymerase, it cannot function as a polymerase, because it lacks the essential active site residues. The computational analysis also identified a substantial disorder within the C-terminal region, suggesting its participation in protein-protein interactions. The subsequent experiments in the Prof. V. Mizrahi's lab directly confirmed computational findings. In turn, a computational model of Rv3395c (Fig. 5) allowed the design of its truncation variants that subsequently were used to map the Rv3395c regions important for its interaction with Rv3394c. A computational model of the DnaE2 polymerase, which was shown to be directly responsible for the mutagenic translesion synthesis (TLS), provided clues as to its functional specialization. Compared with the main replicative polymerase (DnaE1), DnaE2 lacks the C-terminal domain, which mediates interaction with the polIII τ-subunit. The τ-subunit is coordinating leading and lagging DNA strand synthesis during replication. The absence of the τ-interacting domain suggests that DnaE2 is unable to substitute DnaE1 within the replisome. In addition, the absence of key histidine and aspartate residues in the PHP domain of DnaE2 hints at compromised proofreading function. Taken together, these computational results played an important role in revealing individual roles of the DnaE2-Rv3394c-Rv3395c "mutagenic complex" components as well as mapping their interaction regions. Results of the study have been reported in Proceedings of the National Academy of Sciences of the USA [6].

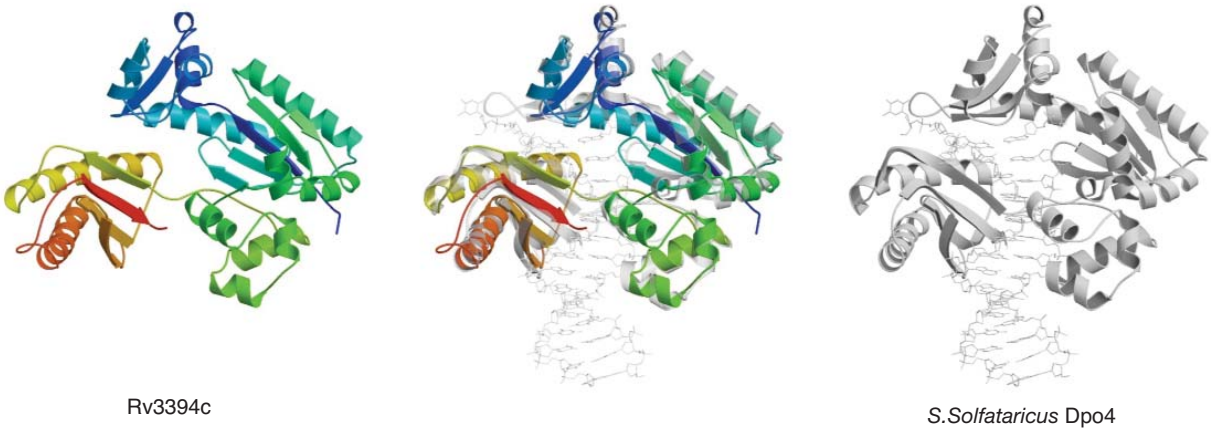

Figure 5. Structurally, MTB ImuB (Rv3394c) is very similar to a Y-family polymerase (Dpo4), but it lacks polymerase active site residues.

International collaboration

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Prof. Valerie Mizrahi, University of the Witwatersrand, Johannesburg, South Africa

Prof. Martin Kupiec, Tel Aviv University, Israel

Dr. Lajos Haracska, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

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1. **Repšys V, Margelevičius M, Venclovas Č.** Re-searcher: a system for recurrent detection of homologous protein sequences. BMC Bioinformatics 2008, 9:296.
2. **Venclovas Č, Margelevičius M.** The use of automatic tools and human expertise in template-based modeling of CASP8 target proteins. Proteins 2009, 77 Suppl 9:81-8.
3. **Margelevičius M, Venclovas Č.** Detection of distant evolutionary relationships between protein families using theory of sequence profile-profile comparison. BMC Bioinformatics 2010, 11:89.
4. **Margelevičius M, Laganeckas M, Venclovas Č.** COMA server for protein distant homology search. Bioinformatics 2010, 26(15):1905-6.
5. McCauley MJ, Shokri L, Sefcikova J, **Venclovas Č**, Beuning PJ, Williams MC. Distinct double- and single-stranded DNA binding of E. coli replicative DNA polymerase III α subunit. ACS Chem Biol 2008, 3(9):577-87.
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7. **Laganeckas M, Margelevičius M, Venclovas Č.** Identification of new homologs of PD-(D/E)XK nucleases by support vector machines trained on data derived from profile-profile alignments. Nucleic Acids Res 2010, doi:10.1093/nar/gkq958.
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BOOK CHAPTER

1. Barsky, D., Lawrence, T.A. and **Venclovas, Č.** How proteins slide on DNA. In Williams, M. C. and Maher, L. J. III (eds.), Biophysics of DNA-Protein Interactions. Springer Science+Business Media, LLC., In press.

DNA Sequencing Center

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DNA Sequencing Center (SC) of the Institute of Biotechnology (IBT) is successfully running since March 27 of 2003. 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. The Center is equipped with the Applied Biosystems 3130xl Genetic Analyzer 16-capillary automated DNA sequencer that yields 700 to 1000 bases per template. It 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 2-3 days after the receipt of samples. Sequencing of the larger samples may take longer.

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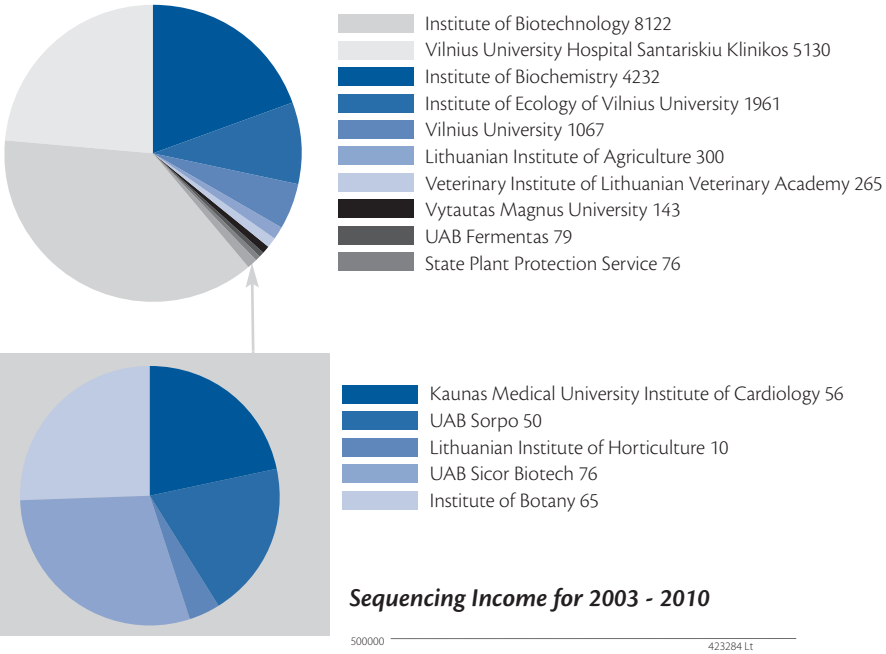

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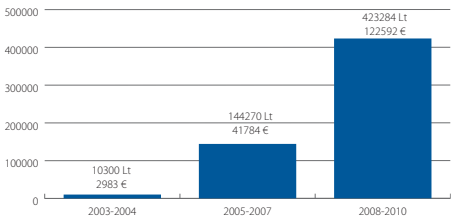

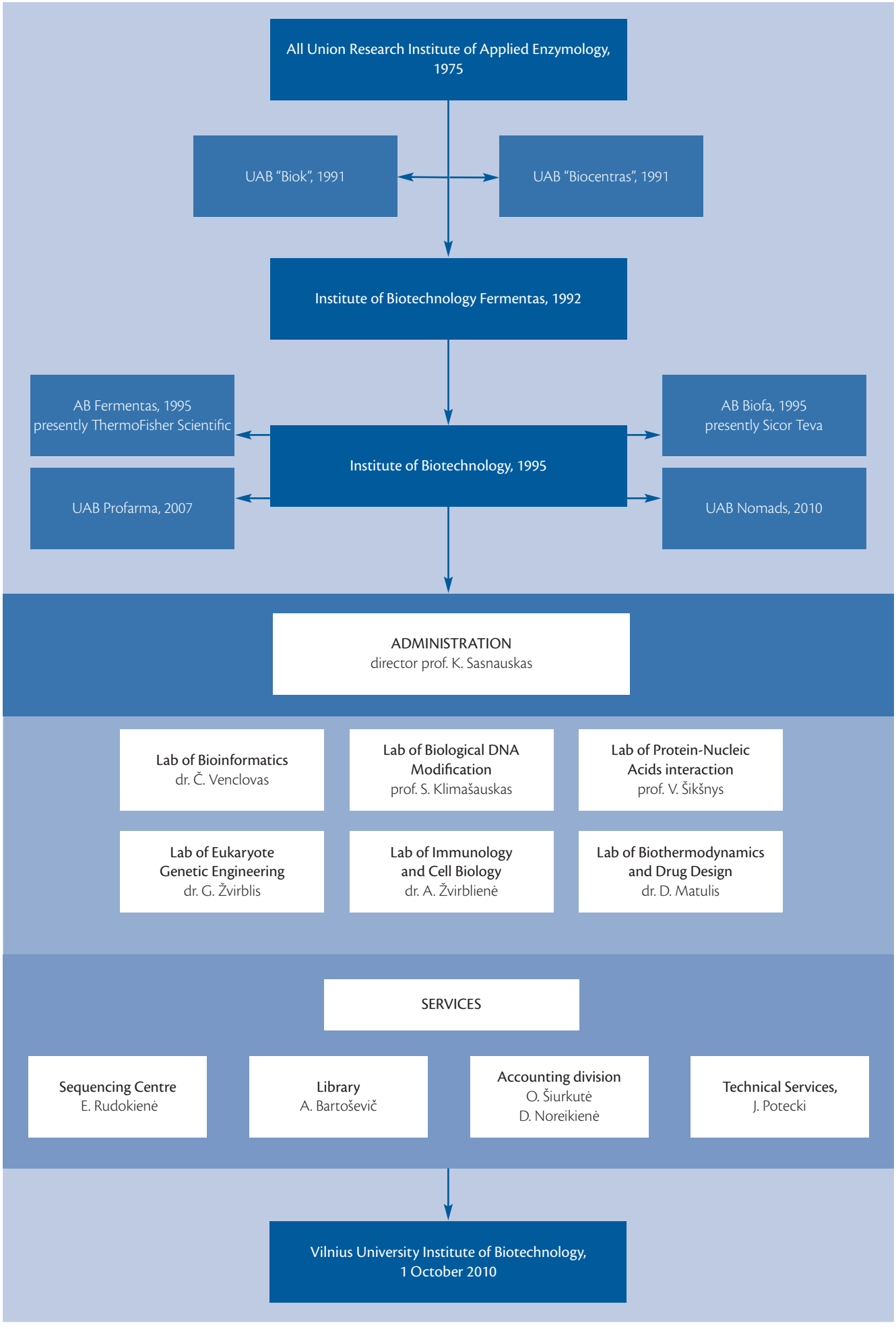
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