Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Institute of Microbiology of the CAS, v. v. i.

The Institute of Microbiology of the ASCR, v. v. i., has presently more than 640 employees (517 full-time equivalents) and belongs to the main institutions in the Czech Republic involved in the basic microbiological research. Basic research in biochemistry, physiology and molecular genetics of bacteria, yeasts, filamentous fungi and microscopic algae and immunological research are the main topics. In addition to the basic research, the Institute is involved in a number of biotechnological applications.

Under Act No. 341/2005 Coll. the legal status of the Institute of Microbiology of the Academy of Sciences of the Czech Republic was transformed from a state contributory organization into a public research institution (abbreviated v. v. i.) from January 1, 2007. This transformation provides a higher level of autonomy and opens new possibilities for economical activities. **Economical activities** of the Institute include the production, trade and services in the fields of biology, chemistry and medical sciences and enable efficient uses of available capacities of biotechnology pilot plants in Prague and Třeboň.

The Institute has 24 research laboratories as basic scientific units and three core facilities (Center for DNA Sequencing center, Center Cytometry and Microscopy, and Biotechnological Pilot Plant). The structure of the Institute is dynamic, the number of laboratories is not constant; within the evaluated period some laboratories were, based on the internal evaluation results, closed or merged, on the other hand, new laboratories were formed.

Publication activity within the evaluated period **has been growing**. Both the number and quality continuously increase. The number of refereed articles within the evaluated period 2010-2014 was by 25 % higher as compared with the last international evaluation in 2009 (944 publications as compared to 744) and the quality measured by IF increased from 2.926 to 3.598 (given as a mean of five-year period). Importantly, papers in high quality journals (*Proc. Natl. Acad. Sci., USA, Plant Cell, The ISME J., Nucleic Acids Res., PLos Pathogens, PLoS Genetics, Mol. Biol. Evol.*) are more and more frequent including those published solely by the Institute's authors. Moreover, our colleagues are frequently invited to write review papers to highly ranked journals (e.g *Chemical Society Reviews, Advanced Drug Delivery Reviews*) and book chapter.

We are succeeding to upgrade continuously the **research equipment** and nowadays the Institute is well equipped by top mass spectrometers, NMRs, flow cytometers, microscopes, sequencers *etc.* Moreover, **unique technologies** for breeding of germ-free animals and culturing of microorganisms or algae are available.

Consistent internal **evaluation mechanisms** are permanently used and adequate conclusions are always made. Within the evaluated period two laboratories were closed or merged, in some cases the head of the laboratory was replaced. It should be

mentioned that the results of the internal evaluation fully correspond to the results of the last international evaluation 2005-2009. All scientists undergo regular attestations at five-year intervals. On the other hand, there are numerous stimulatory activities – best original and review papers are awarded every year. A financial premium is given to PhD students who are first authors on original papers.

The Institute has a long-lasting and fruitful **collaboration with Czech universities** based on official Association Agreements. The Institute´s scientists help undergraduate and PhD students to fulfill their potential and act as supervisors of both diploma and dissertation theses. More than one hundred PhD students are trained in the accredited doctoral programs.

The Institute has a broad **international collaboration** based on research projects or bilateral agreements. It organizes scientific congresses, conferences, symposia, seminars, and other national and international meetings in the Czech Republic and abroad.

The Institute is involved in several EC Structural Funding Projects. The Třeboň Department of Phototrophic Microorganisms of the Institute of Microbiology gained a support of 133 mil. Kč (approx. 500,000 €) from the Operational Programme "Research and Innovation for Development" led by the Czech Ministry of Education, Youth and Sports for the implementation of project Algatech (construction and operation of a new scientific research Centre of algal biotechnologies; www.alga.cz). The implementation phase began on January 1, 2011 and aims to develop new cultivation equipment and algal biomass processing practices for the production of biofuel, animal feeding, food supplements and for the isolation of valuable substances. The project also focuses on research in the area of photosynthesis, the development of new measuring devices and the education of students in these areas.

The Project activities are divided into scientific research and investments. The investment activities of the project have focused on the reconstruction and completion of additional buildings to the original historical building of the Opatovický mill (Opatovický mlýn). Biotech labs have been renovated and a new technology hall has been built. The original mill room has been rebuilt into laboratory facilities for molecular biology and analytical methods. Another important part of the investment activities is the purchase and installation of necessary modern technology.

The scientific research activities are divided into three research programs. Activities of the first research program aim at the development of a technical and biotechnological base and the methodological approaches in a large-scale production of micro-organisms - microalgae (photosynthetic bacteria, cyanobacteria and green algae) under autotrophic and heterotrophic growth conditions. Activities of the second research program focus on research in photosynthesis, the development and testing of new optical methods applied in ecophysiology of phytoplankton and for determining the primary production in freshwater environments. Activities of the third research program focus on enhancing the theoretical knowledge of heterotrophic algal biomass production, which has required a complete innovation of the technological base for heterotrophic microalgae cultivation. This process will further lead to the overall optimization of the fermentation process. The main goal will be to attain the required

chemical content of biomass and to develop methods for its complex processing while focusing on the isolation of valuable substances.

Researchers working at the Institute of Microbiology in Třeboň are actively involved in teaching bachelor's, master's and doctoral degree programs and each laboratory at the Třeboň workplace is a place of study of numerous doctoral students. Therefore, the management of the Centre is able to choose among successful students (in Master's and Ph.D. degree programs) and offer them the opportunity to participate in scientific activities and projects of the Center.

Two Czech-Polish regional projects submitted by the Laboratory Gnotobiology in Nový Hrádek have been granted to support both the personnel and investment costs (new flow cytometer).

The Laboratory of Characterization of Molecular Structure succeeded in the Operational Program Prague – Competitiveness and the Institute thus strongly benefits from the new instrumentation in total value of approx. 400,000 €.

Last but not least, the Institute is involved in project BIOCEV (www.biocev.eu) as a joint project of six institutes of the Academy of Sciences of the Czech Republic (Institute of Molecular Genetics, Institute of Biotechnology, Institute of Microbiology, Institute of Physiology, Institute of Experimental Medicine, and Institute of Macromolecular Chemistry) and two faculties of Charles University in Prague (Faculty of Science and 1st Faculty of Medicine). The project's goal is to establish European Centre of Excellence in biomedicine and biotechnology. Related program projects supporting BIOCEV have been already successfully closed and enabled to recruit new post-doc from abroad.

Twenty-four evaluated research laboratories of the Institute can be clustered into seven major research directions:

- Microbiology
- Biology of the Cell and Bioinformatics
- Structural Biology
- Biogenesis
- Ecology
- Phototrophic Microorganisms – Center Algatech
- Immunology

Microbiology concentrates on research of molecular biology and genetics of prokaryotic microorganisms. Regulations of gene expression, effect of internal and external conditions on cell functions, as well as molecular principles of bacterial pathogenicity are investigated. The results obtained open new ways towards new industrial and biomedical applications.

Biology of the Cell and Bioinformatics concentrates on research of molecular biology and genetics of eukaryotic microorganisms and cells. Regulation of gene expression, cell differentiation, and effect of internal and external conditions on cell

functions, mechanisms of cell aging, and significance of cytoskeleton apparatus in cell division are investigated. Last but not least, computer modelling of biological processes in the cell provides bioinformatics support for the measured data.

Structural Biology laboratories combine three analytical tools for the characterization of molecular structure: mass spectrometry, nuclear magnetic resonance spectroscopy and microscopy.

Biogenesis - the main topics of interest are physiology and genetics of mycelial actinomycetes producing secondary metabolites and genetics, physiology and biotechnology of filamentous fungi. Other projects include antibiotic resistance of bacteria, biotransformation of natural compounds and enzyme technologies. The laboratories tightly collaborate with the biotechnological pilot plant, which is involved in process engineering and optimization of microbial fermentations and production of biologically active compounds on a larger scale.

Ecology research program covers the ecology, physiology and biochemistry of microorganisms in the environments, especially in soils, plant litter, decomposing wood or in association with plants, as well as it includes complex physiological, biochemical and genetic characterization of fungal enzyme systems capable of biodegradation of pollutants such as aromatic hydrocarbons. Interactions between mycorrhizal fungi and soil organic matter are also investigated.

Immunology. The origin and development of immune response, functional characterization of components of the immune system and regulation of immune response are studied. Important results were obtained in the research of autoimmune and cancer diseases. Laboratory of Gnotobiology located in Nový Hrádek in East Bohemia uses the unique model of germ-free animals for the study of relationships between microorganisms and host.

Center Algatech is located at the Opatovice pond in Třeboň in South Bohemia and its research program includes the study of photosynthetic microorganisms, *i.e.* algae, cyanobacteria and other photosynthetic bacteria. One of the laboratories is involved in the technological production of algae, its optimization, and processing of algal products, as well as in development of different methods for the utilization of algal biomass.

In conclusion, the Institute of Microbiology is internationally competitive, in some fields is a real leader at least at the European level and has a perspective for a future growth.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory for Biology of Secondary Metabolism

Laboratory was established in 2005. Compared to the first evaluation period 2005-2009, when the laboratory was evaluated 1.5, **both quantitative and measurable qualitative scientific outputs significantly increased:** 36 results in ASEP, 31 of them in impacted journals (compared to 20 in last period), moreover 2 patents, 2 book chapters and 1 scientific book edited by member of the team. Even more **significant progress is apparent when papers with our major participation are considered** (first and/or corresponding author from the group). In 2010-2014 there were 18 such publications in journals with average impact factor (IF) 3.6 (compared to 11 papers in journals with average IF 2.2 in previous period).

A substantial majority of these publications with an essential participation of laboratory is represented by papers on the main topic of the group, the biosynthesis of secondary metabolites, especially lincosamide antibiotics and biosynthetically related compounds with a variable spectrum of biological activities and also resistance to antibiotics. **A mutual thematic linkage of the publication outputs synergistically enhances their overall impact**. The group is actively cooperating with other groups within the institution as well as at the national and international level in two planes: the topical and methodological (structural biology methods, NGS, other omics methodological approaches). These collaborations aim to **conceptual development of the solved topics in prospective trends using the advanced and relevant methodological instruments** including the complex ones, with the prospect of participation in the interdisciplinary projects at both the national and international level.

A) Scientific outputs

The selected outputs discussed below are listed in D) References and sorted into five clusters A1-A5. The laboratory has produced 12 outputs on biosynthesis of lincosamides and biosynthetically related secondary metabolites (clusters A1, A2) and antibiotic resistance (A3), all with major participation of team members. Remaining 7 selected outputs concern other topics related to secondary metabolism (A4) or to processing peptidases of mitochondrial type as a potential target for antiprotozoal agents (A5). P stands for outputs of the group from previous evaluation period relevant for continuity documentation.

A1. Biosynthesis and biotechnology of lincosamide antibiotics: Lincosamide synthetase is a unique hybrid system combining NRPS and non-NRPS elements.

Lincosamides are small but clinically important (lincomycin, clindamycin) group of **antibacterial agents, some synthetic derivatives exhibit also significant antiprotozoal (antimalaric) activity**. Recent papers published mainly by three groups (USA, China and our group) reveal moreover **uniqueness and fascinating features of lincosamide biosynthesis** (coupling of biosynthesis with mycothiol-dependent detoxification system [**A1/1**; Zhao et al., *Nature* 518, 115-119, 2015] and unique mechanism of sulfur atom incorporation into the lincosamide structure [Melançon III Ch. E., *Nature* 518, 45–46, 2015]). The lincosamide

biosynthesis thus became a **highly attractive model documenting general principles of molecular evolution of secondary metabolites**.

The final lincosamide scaffold arises from condensation of two biosynthetic precursors, amino acid (L-proline or its derivative) and an amino sugar (octose). The condensation is catalyzed by **lincosamide synthetase (LS)**, a unique hybrid system combining elements of NRPS and non-NRPS [**A1/1; A1/2**]; in case of lincomycin also both precursors, the octose and 4-propyl-L-proline (PPL), are synthesized in unique or highly specialized biosynthetic pathways, which were recently solved or substantially revised: the specialized octose pathway was solved by USA group [Lin C.I. et al: *J Am Chem Soc* 136: 906-909, 2014; Sasaki E. et al.: *J Am Chem Soc* 134: 17432-17435, 2012] the PPL pathway has been proposed in 1984 [Brahme M. et al.: *J Am Chem Soc* 106: 7878-7883, 1984] but now is being completely revised by our group [**A2/5**] (see part A2). **Our group thus substantially contributed to elucidation of two out of three key processes in lincosamide biosynthesis**.

The significant general output [**A1/1**] was preceded by biochemical papers describing kinetic parameters of enzymes involved in condensation of two precursors **[A1/2]** or in following biosynthetic steps [**A1/3; A1/4**]. Paper [**A1/2]** has identified and characterized the first subunit of LS, a stand-alone adenylation domain (A-domain) determining the substrate specificity of the whole LS complex. We demonstrated by comparative analysis of A-domains from related biosyntheses of two lincosamides lincomycin and celesticetin the adaptation of LS to accept newly emerged unusual precursor PPL aroused from specialized pathway (see part A2). This output explains at the molecular level the feasibility of the mutasynthetic preparation of more efficient lincomycin derivatives [**A1/5** and patent **A1/6**] and **reveals the potential of the LS system for further biotechnological applications**. The relaxed substrate specificity of following postcondensation methylation step (proved biochemically [**A1/3**] and even based on **solved crystal structure of N-methyl transferase** [**A1/4**]) confirms the suitability of the system for utilization of modified precursors.

A2. 4-Alkyl-L-proline derivative precurcors: <u>The specialized biosynthetic pathway shared by three groups of biotechnologically significant natural products was substantially revised.</u>

The alternative approach to the above mentioned mutasynthetic preparation of more potent lincomycin derivative could be **genetic modification of the amino acid precursor biosynthetic pathway** in the producing strain aiming to direct incorporation of modified precursor. The highly related amino acid precursors (4-alkyl-L-proline derivatives, **APD**s) are incorporated into **three structurally different and functionally diversified groups of natural compounds - pyrrolo[1,4]benzodiazepines (PBDs) with antitumor activity, bacterial hormone hormaomycin and antibiotic lincomycin**. The APDs are synthesized from L-tyrosin in evolutionary related pathways. General concept of the APD biosynthetic pathway was postulated 30 years ago [Brahme M. et al.: *J Am Chem Soc* 106: 7878-7883, 1984]. For lincomycin model, the functional testing of LmbB2 [**A2/1**] and LmbB1 [P1] enzymes confirmed the original Brahme´s concept for two initial biosynthetic steps, however, remaining four predicted steps were found to be contradictory with recent knowledge of related gene clusters and structures of final products. So far six gene clusters coding for biosynthesis of APD incorporating compounds are available (including the porothramycin one recently identified and characterized by our group [**A2/2**]). **We proposed a new biosynthetic scheme of the APD special pathway based on analysis of production profiles of lincomycin producing strain inactivation mutants together with in vitro testing of relevant recombinant enzymes** [**A2/3**]. The functions of three out of four remaining

enzymes had to be revised: for methyltransferase LmbW an alternative natural substrate was proposed while LmbX and LmbA putatively catalyze substantially different reactions. Particularly challenging seems to be the cleavage of C-C bond putatively mediated by action of γ-glutamyltrasferase LmbA employing transient attachment of γ-glutamyl to the substrate.

Our results obtained on lincomycin model are valid also for other APD incorporating compounds, mainly PBDs, including extended biotechnological implications.

A3. Antibiotic Resistance: <u>The new ribosom-targeted mode of action of ARE group of ABC proteins was proposed.</u>

After return from the postdoc fellowship in the group of Dr. Hong at University of Cambridge, Gabriela Balikova-Novotna started to form new internal team composed of postdoc Vladimir Vimberg, two technicians on part-time job (Marketa Koberska, Jana Vesela), one PhD student (Jakub Lenart) an two undergraduate students. **This newly established subgroup headed by G. Balikova Novotna is focused on mechanism of action of glycopeptide and MLS (Macrolide-Lincosamide-Streptogramin) group of antibiotics and resistance to them** and finished two papers published in AAC at the end of evaluation period. Jakub Lenart's paper extends results of two papers of G. Balikova Novotna from previous evaluation period [P2, P3] in which we described new form of Vga(A) protein conferring shifted resistance pattern from streptogramins to lincosamides in coagulase-negative staphylococci. Based on detailed mutation analysis of individual residues of putative linker region of Vga(A) together with analogy to the recently described function of the homologous ABC-F protein EttA as a translational factor, **we postulated new hypothesis of mechanism of action of the whole ARE group (<u>a</u>ntibiotic <u>re</u>sistance) of ABC proteins [A3/1] rather by interaction with the ribosome than by generally supposed efflux mechanism.** ARE group consists of wide spectrum of significant antibiotic-resistance determinants, including Msr(A), one of major factors conferring resistance to macrolides. In Vladimir Vimberg's paper **[A3/2] we demonstrated the interference of Msr(A) function (resistance to telithromycin) with function of ClpX chaperon in *Staphylococcus aureus.*** The ClpX relevance was revealed by resequencing of telithromycin resistant *S. aureus* genome **(application of Next Generation Sequencing methodology)** and confirmed by molecular genetic methods.

Oral microbiome in periodontitis: dynamics and response to antimicrobial agent

The NGS was also employed in the study of oral microbiome (OM) associated with health and periodontitis **[A3/3]**. Deep taxonomic characterization of individual OM in time as well as comparison of individual OMs within tested groups and among them **revealed the dynamic changes in the OM composition leading from healthy state to periodontal disease resembling the ecological succession. The OM response to administration of antimicrobial agent was evaluated.** Our results bring the **opportunity for early diagnosis** of individuals in risk of periodontitis together with a tool for **monitoring of treatment**.

A4. Other publication outputs relevant to production of secondary metabolites

Two papers concern general methodological tools for search of natural compounds in cultivation media [**A4/1**] and monitoring of environmental microbiome [**A4/2**]. Other two papers concern biosynthesis of coumermycin A1, antibiotic partially related to lincosamides

[**A4/3**] and biosynthesis of precursor of monensin A, one of model natural compounds studied in preceding period [**A4/4**].

A5. Processing peptidases of mitochondrial type: <u>Potential target for antiprotozoal agents</u>.

Mitochondrial processing peptidase (MPP) and related peptidases (e.g. hydrogenosomal processing peptidase from *Trichomonas vaginalis*; HPP; [P4]) have been important experimental models in history of the laboratory, as it opened the cooperation with group of Eva Kutejova, which provided **transfer of expertise in structural biology and protein science to main topic of our lab** (see in results [**A1/2; A1/3; A2/1]**) and mainly **[A1/4**] where the structure of protein involved in lincomycin biosynthesis was solved).

Processing peptidases of mitochondrial type from parasitic microrganisms are relevant to our main research topic as a potential target for antiprotozoal drugs. While in organelles of *Trypanosoma brucei* surprisingly a regular type of MPP was found [**A5/1**], in hydrogenosoms of *Trichomonas vaginalis* a related, but not identical type of processing peptidase (HPP) was identified representing thus potential target for specific antiprotozoal agents [P4**].** The preparation of diffracting HPP crystal was successful last year and very recently we also solved the crystal structure of the protein (paper in preparation). The detailed mechanism of MPP and HPP function was studied by several methodological approaches [**A5/2; A5/3**] to **reveal particular structural elements distinguishing both related proteins and thus representing potential targets for antiprotozoal agents**.

B) <u>Collaborations</u>

National:

Doc. V. Žlábek, Faculty of Fisheries and Protection of Waters, University of South Bohemia (**GACR 15-04258S, 2015-2017**)

Dr. V. Adámková, General University Hospital in Prague (**AZV 15-28807A 2015-2018**)

Prof. J. Dušková, Institute of Clinical and Experimental Dental Medicine of First Faculty of Medicine of Charles University and General University Hospital in Prague ([**A3/3**], **AZV 15-28142A 2015-2018**)

Dr. T. Etrych, Institute of Macromolecular Chemistry ASCR (newly established collaboration of GBN group aimed on study of polymer conjugated glycopeptides)

International:

Dr. E. Kutejová, J. Bauer, Laboratory of Biochemistry and Protein Structure, Institute of Molecular Biology SAV, Slovakia [**A1/3; A1/4**]

Dr. D. Ulanova, Center for Advanced Marine Core Research, Kochi University, Japan ([**A1/1**], preparation of a common grant project)

Dr. H.J. Hong, University of Cambridge, UK (3 years postdoc G. Balíková Novotná)

Dr. G. Challis, University of Warwick, UK (1 year postdoc Z. Kameník)

Dr. M.J. Horsburgh, Institute of Integrative Biology, University of Liverpool, UK (V. Vimberg 4 month, *S. aureus* mutagenesis and analysis of genome resequencing data **[A3/2]**)

Prof. T. Langer, Institute of Genetics, University of Cologne, Germany (A. Samad 6 months)

Prof. P. Herczegh, University of Debrecen, Hungary (study of resistance of *S. aureus* to new teicoplanin derivatives)

C) Development of personal capacity

In the evaluated period the personal composition of the team was stabilized in both aspects: the age structure and professional skills. Three persons (> 65 years in Chart in part 1) with part-time contracts until 2014 will fully retire during 2015 or changed to 0.1 FTE (emeritus professor J. Spizek, former director). The team now possesses a pyramidal composition with 5 experienced scientists, 4 postdocs and students of PhD and undergraduate levels. The whole group is supported by 2 graduated technicians. The team acquired two specialists in essential, formerly outsourced fields ("T" in previous SWOT analysis), experienced in organic chemistry (R. Gažák) and instrumental analysis (Z. Kameník, former PhD student, returned after 1 year postdoc fellowship in an excellent group of Greg Challis at the University of Warwick). A part-time team member Eva Kutejova is simultaneously a leader of Laboratory of Biochemistry and Protein Structure, Institute of Molecular Biology SAV (Slovakia). Thanks to her contribution the group is now well settled in the field of protein science **[A1/4].**

The group participates in the project Center of excellence BIOCEV in Vestec in program 3.5. Natural compounds: structure and function. Two key team members (Janata, Najmanová) were managing two BIOCEV synergistic projects OPVK (2012-2014) aimed to formation and training of excellent scientific teams of Institute of Microbiology for BIOCEV (with participation of 11 groups from the whole institute). Temporarily in the period of projects preparation and start (2011-2012) it caused a remarkable gap in their scientific activity and subsequently also the drop of number of scientific outputs from the whole group in this period. However, in following period this activity brought the group the expected benefits in the form of repatriation of **G. Balíková Novotná** from 3 years postdoctoral stay at University of Cambridge as a **leader of new team prepared for BIOCEV** (supported by OPVK 20.0055) and **integration of three postdocs selected by international tender** (supported by OPVK 30.0003) Vladimir Vimberg (strengthened the arising group of G. Balíková Novotná), Abdul Samad and Zdenek Kameník (with perspective for further development of the group in the field of metabolomics).

D) References

Selected papers published by evaluated team in 2010-2014 (results in ASEP, short reference formate) or aroused from results of evaluated period (not yet in ASEP, full reference formate). The authors afiliated to the group are underlined.

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A1/2 Kadlčík S., Kučera T., Chalupská D., Gažák R., Koběrská M., Ulanová D., Kopecký J., Kutejová E., Najmanová L., Janata J. (2013) *PLoS One* 8(12): e84902.

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A1/5 Ulanová D., Novotná J., Smutná Y., Kamenik Z., Gažák R., Šulc M., Sedmera P., Kadlčík S., Plháčková K., Janata J. (2010) *Antimicrob Agents Chemother* 54(2), 927-930.

A1/6 Ulanová D., Novotná J., Smutná Y., Kameník Z., Gažák R., Janata J., Kopecký J a Spížek J.: The method of biotechnological preparation of lincomycin derivatives and its using CZ patent č. 302256 (2010).

A2/1 Novotna J., Olsovska J., Novak P., Mojzes P., Chaloupkova R., Kamenik Z., Spizek J., Kutejova E., Mareckova M., Tichy P., Damborsky J., Janata J. (2013) *PLoS One* 8(12): e79974.

A2/2 Najmanova L., Ulanova D., Jelinkova M., Kamenik Z., Kettnerova E., Koberska M., Gazak R., Radojevic B., Janata J. (2014) *Folia Microbiol.* 59: 543-552.

A2/3 Jiráčková P., Gažák R., Kameník Z., Najmanová L., Kadlčík S., Steiningerová L., Novotná J., Kuzma M., Janata J.: Revision of the biosynthetic pathway for 4-alkyl-L-proline precursors of pyrrolobenzodiazepines, lincomycin and hormaomycin. (2015) *Chem. Biol.* (submitted)

A3/1 Lenart J., Vimberg V., Vesela L., Janata J., Novotna G. B. (2014) *Antimicrob Agents Chemother* doi:10.1128/AAC.04468-14

A3/2 Vimberg V., Lenart J., Janata J., Novotna G. B. (2015) *Antimicrob Agents Chemother* doi:10.1128/AAC.04367-14

A3/3 Sabová L., Janatová T., Dušková J., Broukal Z., Najmanová L., Janata J.: The taxonomic composition of oral microbiome enables early diagnosis of patients in risk of periodontitis development. (2015) (prepared for publication in *PLoS Pathogens*)

A4/1 Kameník Z., Hadacek F., Marečková M., Ulanová D., Kopecký J., Chobot V., Plháčková K., Olšovská J. (2010) *J. Chromatograph. A* 1217(51), 8016-8025.

A4/2 Sagova-Mareckova M., Omelka M., Cermak L., Kamenik Z., Olsovska J., Hackl E., Kopecky J., Hadacek F. (2011) *Appl Environ Microbiol* 77, 7560.

A4/3 Novotna J., Gust B., Kulik A., Spizek J., Heide L. (2013) *J Ind Microbiol Biotechnol* 40(8):915-25.

A4/4 Kyselková M., Janata J., Marečková M. and Kopecký J. (2010) *Arch Microbiol* 192(3), 195-200.

A5/1 Mach J., Poliak P., Matušková A., Žárský V., Janata J., Lukeš J., Tachezy J. (2013) *Genome Biology and Evolution* 5(5), 860-865.

A5/2 Dvořáková-Holá K., Matušková A., Kubala M., Otyepka M., Kučera T., Večeř J., Heřman P., Parkhomenko N., Kutejova E., Janata J. (2010) *J. Mol. Biol.* 396 (5), 1197 – 1210.

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P2. Novotna, G., Adamkova, V., Janata, J., Melter, O., Spizek, J. (2005) Prevalence of resistance mechanisms against macrolides and lincosamides in methicillin-resistant coagulase-negative staphylococci in the Czech Republic and occurrence of an undefined mechanism of resistance to lincosamides. *Antimicrob Agents Chemother* 49: 3586-3589

P3. Novotna, G., Janata, J. (2006) A new evolutionary variant of the streptogramin A resistance protein, vga(A)(LC), from Staphylococcus haemolyticus with shifted substrate specificity towards lincosamides. *Antimicrob Agents Chemother* 50: 4070-4076

P4. Šmíd O., Matušková A., Harris S. S., Kučera T., Novotný M., Horváthová L., Hrdý I., Kutějová E., Hirt P. R., Embley T. M., Janata J., Tachezy J. (2008) Reductive Evolution of the Mitochondrial Processing Peptidases of the Unicellular Parasites Trichomonas vaginalis and Giardia intestinalis. *PLoS Pathogens* 4 (12), 1-8

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Fungal Genetics and Metabolism

Summary of the research results

The laboratory concentrates on several microorganismal groups which are important for humans including plant pathogens (ergot fungi), human and animal pathogens (*Aspergillus*, dermatophytes, *Pseudogymnoascus*) and fungi with high potential of production of new enzymes or biologically active metabolites. Natural products are typically produced by microbes living in strong competition, which includes soil or endophytic fungi and microorganisms living in symbiotic relationships with other organisms (plants, insects). All these habitats were studied and newly discovered species analyzed in detail. In each group, the ecology, evolution and diversity is studied together with taxonomy and biological activity of the natural compounds. The laboratory consists of taxonomists, molecular geneticists, microbiologists and specialists in organic chemical separation and analysis. The laboratory is a prestigious and world-wide recognized centre for study of *Claviceps*, *Geosmithia, Aspergillus* and dermatophytic fungi.

The most important research results

Evolution, diversity and taxonomy of the genus Claviceps. (S. Pažoutová, M. Kolařík, K. Pešicová, M. Chudíčková, J. Olšovská).

The laboratory is a world-wide recognized centre for study of *Claviceps* and a unique collection of *Claviceps* species (Culture collection of Clavicipitaceae, member of WFCC) is maintained there. We finished the revision of ergot fungi from warm season grasses from the entire world. Eight new species, often infecting economically important pasture grasses, were described (Pažoutová et al. 2011). Dr. Sylvia Pažoutová spent his professional life with work on ergot. She suddenly passed away in Sept. 2013 and we are continuing in here work. The long time (over 30 years) study of the cryptic species inside *C. purpurea*, which is the most important ergot species, resulted in complex revision of this fungus (Pažoutová S, Pešicová K, Chudíčková M, Šrůtka P, Kolařík M, 2015. Delimitation of cryptic species inside *Claviceps purpurea*. Fungal Biology 119: 7-26). We collaborated with G.N. Odvody (Texas A&M AgriLife) and D. Frederickson which provided most of the ergot specimens from Africa and America.

Diversity, ecology and evolution of insect symbiotic fungi. (M. Kolařík, S. Pažoutová, T. Veselská, M. Kostovčík, M. Chudíčková)

The effect of the host tree, location and host insect on the spectrum of fungi associated with siricid woodwasp (*Xiphydria* spp.) was revealed (Pažoutová et al. 2010) and new species of insect associated *Daldinia* was described, together with the characterization of its secondary metabolites (Pažoutová et al. 2013). Insect vectored fungi are known to interact with natural inhabits of healthy phloem. Thus we surveyed endophytic fungi of trees and described new genus *Liberomyces* (Pažoutová et al. 2012). In our group, a new type of symbiosis between

Geosmithia and bark beeltes was discovered and the complex characterization of this symbiotic complex is one of the leading projects in our lab. We found that these fungi live also as obligate ambrosia fungi (Kolařík et Kirkendall 2010), can be serious pathogens (Kolařík et al. 2011, Hadziabdic et al. 2014) and are significant associates of some conifer attacking bark beetles (Jankowiak et Kolařík 2010b, Kolařík et Jankowiak 2013, Jankowiak et al. 2014). We also showed that these fungi exchange genome segments within each other and with other plant pathogenic fungi (Frascella et al 2014, Bettini et al. 2014). The first results of evolutionary ecology of these fungi, based on genome size comparisons were published (Veselská et al. 2014, Veselská T, Kolařík M, 2015. Application of flow cytometry for exploring the evolution of *Geosmithia* fungi living in association with bark beetles: the role of conidial DNA content. Fungal Ecology 13: 83-92). We also firstly characterized spectrum of the bacteria in the mycangia of ambrosia beetles (Kostovcik M, Bateman CC, Kolarik M, Stelinski LL, Jordal BH, Hulcr J, 2015. The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. Isme Journal 9: 126-138). The study of the other bark beetle associated fungi was focused on ophiostomatoid fungi (Jankowiak et Kolařík 2010).

We collaborated with R. Jankowiak (Agricultural University of Cracow) which provided all isolates and other data from Poland. L. R Kirkendall (University of Bergen) provided bark beetle samples from tropics. Jiří Hulcr (University of Florida) was responsible for the study of bacterial symbionts of ambrosia beetles. M. Kolařík contributed by isolates, statistical analyses and manuscript preparation in papers which were conducted together with team of A. Scala (University of Florence) and D. Hadziabdic (University of Tennessee). Ned Tisserat team (Colorado State University Fort Collins) contributed by isolates of *Geosmithia morbida*. Jiří Holuša and P. Šrůtka (Czech Agricultural University) provided woodwasp samples. Group of M. Stadler (Helmholtz Centre for Infection Research, Germany) mostly contributed by the characterization of *Daldinia* secondary metabolites.

The taxonomy and diversity of the genus Aspergillus. (V. Hubka, M. Kolařík, A. Nováková, Z. Dudová, F. Sklenář)

Species of *Aspergillus* are molds with great importance for human and animal health, agriculture and biotechnology. The large taxonomic revision of section *Aspergillus* and *Fumigati* was completed (Hubka et al. 2013, Hubka et al. 2014, Nováková et al. 2012, Nováková et al. 2014, Hubka V, Novakova A, Kolarik M, Jurjevic Z, Peterson SW, 2015. Revision of *Aspergillus* section *Flavipedes*: seven new species and proposal of section *Jani* sect. nov. Mycologia 107: 169-208). The published results included taxonomic revisions and typification of previously described species, description of new taxa and characterization of their mycotoxines. The modern identification schemes were created for reliable species identification in praxis. The characterization of the beta tubulin paralogue has great impact in taxonomy not only in *Aspergillus* but for all fungi (Hubka et Kolařík 2012).

These studies were done together with S. Peterson (USDA, Peoria), Z. Jurjevic (EMSL Analytical, Inc., USA) and other collaborators which provided isolates or part of molecular data. Other researchers from the Charles University (A. Kubátová) and other Czech (P. Lysková, M. Skořepová) and Japan (T. Matsuzawa, T. Yaguchi) institutions provided fungal isolates.

Epidemilogy, taxonomy and diversity of human pathogenic fungi (V. Hubka, M. Kolařík, A. Čmoková, Z. Dudová).

Fungi causing mycoses are important pathogens of humans. We focused on the epidemiology, polyphasic taxonomy (molecular data, morphology, physiology, ecology, mating experiments), clinical relevance and antifungal susceptibilities of aspergilli, dermatophytes and non-dermatophytic molds. Since 2011, we are managing the net of clinical mycologists from all parts of the Czech Republic in order to make large scale epidemiological study (2012-2017). The provided isolates were identified and their identity, taxonomy and antifungal susceptibility assessed. The first results of the epidemiological surveys were published (Hubka V, Větrovský T, Dobiášová S, Skořepová M, Lysková P, Mencl K, Mallátová N, Janouškovcová H, Hanzlíčková J, Dobiáš R, Čmoková A, Stará J, Hamal P, Svobodová L, Kolařík M, 2015. Molecular epidemiology of dermatophytoses in the czech republic – two-year-study results. Cesko-Slovenska Dermatologie 89: 167-174) and three new dermatophytes described (Hubka et al. 2013, Hubka et at al. 2014). The revision of clinical *Aspergillus* isolates resulted in discovery of little known or new etiological agents (Hubka et al. 2012). The studies on other pathogenic fungi resulted in description of the new fungal genus *Bradymyces* which is a serious fish pathogen (Hubka et al. 2014).

Our team is bridging the gap between academic laboratories and hospitals. The most important collaborators were M. Skořepová (Dermatology Clinic, Charles University), H. Janouškovcová (University Hospital in Plzeň), P. Hamal and L. Svobodová (University Hospital Olomouc), Karel Mencl (Regional Hospital of Pardubice), Naděžda Malátová (Central Laboratories of the České Budějovice Hospital), P. Lysková (Public Health institute, Prague), R. Dobiáš (Public Health institute, Ostrava) and A. Kubátová (Charles University). Their contributed by providing isolates, clinical data and by conducting of antifungal susceptibility tests. The co-authors of the *Bradymyces* study contributed by providing of isolates, phylogenetic analyses and other observations.

Taxonomic study on other fungi (M. Kolařík, M. Chudíčková)

We collaborated on identification and taxonoic analysis of fungi from habitats with high proportion of the new taxa. These are fungi from highly acidic soils (pH 1-3). Four new genera were described (Hujslová et al. 2013, Hujslová et al. 2014). Other studies on fungi of bioindicative value (eg. fungi from natural, old grown forests) were conducted (Holec et Kolařík 2011, 2013; Holec et al. 2013, Šutara et al. 2014).

Members of our laboratory contribute by molecular identification, phylogenetic analyses and taxonomic descriptions.

New natural products from symbiotic fungi and other microorganisms. (M. Flieger, E. Stodůlková, T. Tylová, J. Olšovská).

During the study of the new or little known fungi, the basic biological activity screening of the crude extracts was done. The endophytic fungus *Biatriospora* sp. and *Geosmithia* spp. was found as potent producer of numerous new and biologically active quinones (Stodůlková et al. 2010, Stodůlková E, Man P, Kuzma M, Černý J, Císařová I, Kubátová A, Chudíčková M, Kolařík M, Flieger M, 2015. A highly diverse spectrum of naphthoquinone derivatives produced by the endophytic fungus *Biatriospora* sp. CCF 4378. Folia Microbiologica 60: 259-267). The set of new compounds was described from the bark beetles symbiotic fungus *Quamalaria cyanescens*. These compounds have strong potential as anticancer drugs (Stodulkova E, Cisarova I, Kolarik M, Chudickova M, Novak P, Man P, Kuzma M, Pavlu B,

Cerny J, Flieger M, 2015. Biologically Active Metabolites Produced by the Basidiomycete *Quambalaria cyanescens*. PLoS ONE 10. 10.1371/journal.pone.0118913, Flieger et al. 2014). Several other methodical studies were published.

We collaborated with cell biologist (J. Černý, B. Pavlů) on test of the biological activity and with specialists from the Charles University or Institute of Microbiology (I. Císařová, P. Man, M. Kuzma, P. Novák etc) which contributed by the detailed structure elucidation of new compounds.

Flieger M, Stodulkova E, Kolarik M, Man P, Cerny J, Cisarova I, Kralova J, Cisarov I, New submerged logs *Quambalaria* (CCM 8372) and (CCM 8373) for producing naphthoquinone dyes including FSK1 and FSK2, used to modulate morphology of mitochondria. Czech patent no.CZ201200129-A3; CZ304335-B6

Diversity and function of cell lipids produced by microorganisms. (T. Řezanka)

Both theoretically and practically important results were obtained in the exploration of the molecular properties of neutral cell lipids, namely triacylglycerols (TAGs), which are being increasingly studied as compounds with a variety of uses in nutrition, medicine, cosmetics, and as highly useful components of biofuels. The research covered identification and analysis of various TAGs in bacteria, algae, cyanobacteria, diatoms, and yeast, and included TAGs with branched fatty acids, odd-chain TAGs, TAGs containing ω-phenyl fatty acids, polyunsaturated TAGs, and their positional isomers, regioisomers and enantiomers, as well as production of structured TAGs. We used a combination of state-of-the-art HPLC and MS techniques to identify and characterize for the first time the properties of a number of molecular species of TAGs.

We also analyzed natural lipid based biosurfactants such as lipopeptides or rhamnolipids. In addition, we analyzed unusual fatty acids such as eicosapentaenoic acid, pivalic acid and mycolic acids. Moreover, the analysis of hopanoids or unusual lipids of extremophilic bacteria was performed and lipidomic profile of snow algae was published.

The research was conducted in close cooperation with the Lab. Cell Biol. of this Institute, and parts of it were performed in cooperation with the Institute of Biotechnology, University of Chemistry and Technology, Prague. The participation of the researchers from the Institute of Botany, Biorefinery Research Centre of Competence and Department of Ecology, Faculty of Science, Charles University, consisted of suggesting the suitable objects for the study, supervising its biological side (the study was otherwise for the most part analytical), interpreting the data and writing up the manuscripts.

A total of 32 articles were published in journals cited in WOS with the sum total impact factor greater than 90 and 2 chapters in books published by Elsevier and one Czech patent.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Structure Characterization

Our laboratory combines three analytical tools for the characterization of molecular structure: mass spectrometry, nuclear magnetic resonance spectroscopy and microscopy. The instrumentation is spread in buildings A, C, U and L on campus. In addition to own scientific research, contractual work is provided for academic and industrial partners (http://ms.biomed.cas.cz/request/users/login.php).

Mass spectrometry group is focused mainly to proteomics, peptidomics and lipidomics fields. Structural biology approaches provided to the community are represented by chemical crosslinking, hydrogen-deuterium exchange and molecular modeling. For biomolecular separation prior to mass spectrometry 1D/2D chromatographic or gel methods are being employed. For molecular 2D visualization imaging mass spectrometry protocols have been developed (MALDI and NALDI) and implemented on 12T FTICR. In late 2015 the new ion mobility spectrometer will be installed.

Nuclear magnetic resonance group operates a four channel 700 MHz instrument with a cryo-probe dedicated to protein analysis (2013). An older (2009) 400 MHz instrument has been dedicated to analysis of low molecular weight natural compounds, their semisynthetic derivatives or biotransformation products. The third, four channel 600 MHz NMR instrument running in HPLC-NMR configuration, is used for metabolomics projects (http://ms.biomed.cas.cz/currentprojects.php). It also can be on-line linked to a Q-TOF mass spectrometer (micrOTOF-Q III).

Microscopy group runs multiple electron as well as optical microscopy equipment. In late 2014 a new scanning electron microscope FEI NanoSEM 450 was installed. It is equipped with CBS detector for high resolution imaging of non-conductive samples and EDAX Octane Plus detector for EDS analysis and elemental mapping. In accord with other laboratory groups extensive service measurements are being carried out for the whole biological campus in Krc as documented by considerable number of collaborations and joint publications.

Mass spectral group represents the strongest laboratory part in terms both of personnel and instrumentation. The laboratory has state-of-the-art mass spectral equipment dominated by 12T SolariX Fourier transform ion cyclotron resonance mass spectrometer. MALDI TOF-TOF instrument (Ultraflex III, BDal, Germany) is also available. The group runs its mycology laboratory designed for work with pathogens up to BSL-2 hazard category and multiple laboratories dedicated to structural biology. The effective instrument sharing with the leading Czech Universities is enabled through part time scientist prof. Karel Lemr (Palacky University in Olomouc), Assoc. Prof. Miroslav Šulc (Charles University in Prague) and Assoc. Prof. Petr Kačer (Institute of Chemical Technology in Prague).

An excellent personal portfolio is documented by **structural biology** subgroup, soon representing an independent research group based in Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University in Vestec (http://www.biocev.eu/). In 2010-2014 structural mass spectrometry subgroup work included mainly the chemical cross-linking and hydrogen/deuterium exchange (HDX) mass spectrometry. In both these fields, software tools allowing data interpretation and visualization were developed. This resulted in one publication describing web based MSTools [1] and creation of two programs – Xlynx and DeutEx (http://ms.biomed.cas.cz/SWD/). Both program packages are actively used by the team members and significantly increased the data processing speed. Other methodological development led to setup of a unique method for non-ionic detergent removal [2], which further allowed for structural study of a true membrane protein, ADP/ATP transporter [3]. While the first publication was mostly done in our lab (90%), the second one was mainly conducted by the collaborating laboratory in France. Petr Man participated at all stages of the project. Further methodological development focused on novel aspartic proteases suitable for protein digestion in HDX MS. We focused on proteases from carnivorous plants *Nepenthes*. First we described extremely efficient and simple protocol for preparation of large quantities on Nepenthesin-1 [4]. This protocol is also a part of US patent application, where the diagnostic use of these proteases is claimed - *US20140186330 A1, Treatment of gluten intolerance and related conditions* (with Petr Man and Hynek Mrazek as co-inventors). In a subsequent study we showed that Nepenthesin-1 is good alternative to commonly used pepsin in HDX MS protocol and the work also provided some general observation about immobilized protease columns and the use of denaturing agents during the digestion [5].

The application of chemical cross-linking and HDX MS was used to study solution structure on NK cell receptor CD161 isoform A and showed surprising differences between the crystal structure and solution structure [6]. In a subsequent study another combination of structural mass spectrometry techniques (cross-linking and ion mobility), in combination with molecular modelling were used to characterize the structure of NKR-P1C molecule in solution [7]. Combination of structural MS techniques was also applied in other studies either from our group [8-10] or from the collaborating laboratories we instrumentally and mentally supported [11-17].

In the field of protein glycosylation the group identified novel protein modification S-glycosylation (collaboration with Massey University, New Zealand, [18]) and studied the role of glycosylation in liver disease in collaboration with Georgetown University [19-21]. In addition to these main areas group members also took part in various proteomic studies [22-24] as well as in projects focused on secondary fungal metabolites [25,26].

The applied research is illustrated by development of ***Method of Surface Modification for the Purpose of Enrichment of Phosphorylated Peptides for Analysis by Desorption/ionization Mass Spectrometry Techniques***. The Czech patent has already been issued in 2012 (No. 303056) and we recently also received positive position from USPTO. The patent application described the construction of a spraying deposition device for the preparation of functionalized surfaces for laser desorption mass spectrometry [27].

Significant results have also been achieved in the field of **biomolecular imaging mass spectrometry** (IMS). The subgroup started with IMS in the Czech Republic in 2009 when imaging ambient techniques were assembled on our previous FTICR mass spectrometer [28]. We soon switched our attention to nanostructure-assisted laser desorption ionization

(NALDI) based on silicon nanowires [29] distributed by Bruker Daltonics (Bremen, Germany) as a matrix-free alternative to MALDI for analysis of small molecules [30]. The silicon nanowire surface was further oxidized and modified with (pentafluorophenyl)-propyldimethylchlorosilane and has gained an immediate analytical reputation. The surfaces soon were identified as useful DESI substrates [31]. Desorption nanoelectrospray (nanoDESI) was developed at Palacky University in collaboration with us [32]. The source was applied in chiral discrimination [33] or anthocyanin analysis [34] and recently technically improved and characterized in detail [35]. In our previous work also the murine tissue sections were imprinted to NALDI targets, tissues and salts washed out and lithographic transfers then visualized in microprobe scanning mode [36]. NALDI imaging was found faster than MALDI IMS due to the absence of the time-consuming matrix deposition step and NALDI images were cleaner as cationization effects were reduced in individual spots. In a more recent study the catalytic oxidative properties of NALDI surfaces were disclosed enabling double bond(s) localization in lipids and unusual peptides [37]. In our most recent work we disclosed the distribution of globotriaosylceramides in Fabry renal murine tissue [38] and dissected lipid degradation processes in human eye lenses [39]. We also described and rationalized the putative surface enhanced mass spectrometry effect with our newer SolariX FTMS system [40]. The head of laboratory has actively participated in EU COST action BM1104 (Mass Spectrometry Imaging: New Tools for Healthcare Research) as a Czech national representative. The collaborative EU work and gained knowledge in state-of-the-art imaging mass spectrometry [41] represents a basis for future expanding into multimodal imaging field.

Nuclear magnetic resonance group does a lot of contractual work (*see the 3-9 form*) but also carries out its own research. The group is engaged with applications of NMR to study larger **biomolecules as well as metabolomics**. An important part of the research activities is represented by **monitoring asymmetric reactions in organic synthesis**. In this project, we collaborate with the Institute of Chemical Technology in Prague. Together, we have a high number of publications in the years 2010 – 2014 concerning the asymmetric transfer hydrogenation of imines [42-55]. We developed a method to follow organic catalytic reactions in NMR tube enabling *in-situ* monitoring [49,51]. Special attention was paid to the study of the mechanism of catalytic hydrogenation using ruthenium catalysts of Noyori's type [44,47,53]. It was shown that the role of base during the reaction is more important than generally assumed. The results suggested that the protonated base formed an associate with the active ruthenium-hydride species, most probably via a hydrogen bond with the sulfonyl group of the complex. It is assumed that the steric and electronic differences among the bases were responsible for the observations monitored by NMR as well as FTICR MS [44].

Structure determination of **microbial secondary metabolites** represents another important part of NMR research activities. New substances like Puwainaphycins F and G isolated from the soil cyanobacterium *Cylindrospermum alatosporum* C24/89 were isolated and characterized [56]. Aeruginosin-865 from terrestrial cyanobacterium *Nostoc* sp. Lukesova 30/93 was also discovered. It poses quite a unique structure: it is the first aeruginosin-type peptide containing both fatty acid and carbohydrate moiety. It is the first aeruginosin found in the genus *Nostoc.* It shows the anti-inflammatory activity without cytotoxic or barrier disruption effect [57].

Electron microscopy group operated rather obsolete equipment in period 2010-2014. On the contrary, this period was characterized by collaborative research supporting the whole

biological campus in Krc [58-63]. The EM group followed two basic directions. The first one represented the characterization of the macromolecular complexes by classical electron microscopy. We used negative staining methods for characterization of recombinant human ameloblastin (AMBN), an intrinsically disordered matrix protein. Electron microscopy revealed that the AMBN high molecular mass self-assemblies were flat ribbon-like supramolecular structures with an average width of 18 ± 4 nm and with a variable size, ranging from tens to hundreds of nanometers in length. Further it was shown that N-terminal of the AMBN is involved in forming macromolecular structures and not the C-terminal [64]. Using similar methodological approach, the *Arabidopsis* Nitrilase-1 (NIT1) has been studied. We have shown that NIT1 in *Arabidopsis* is present in high-molecular-mass polymers and forms filamentous structures similar to bacterial and fungal nitrilases [59]. In the methodological approach we employed here an immuno-detection of NIT1 by specific antibodies coupled to colloidal gold. This allowed us to clearly detect NIT1 polymers and distinct them from other filamentous proteins, mainly of cytoskeletal origin, which were also presented in the system.

The second direction was characterized as studies of biological surfaces. In this relatively broad scope we dealt with the interactions of intestinal bacteria and intestinal mucosa, especially gliadin affected surfaces. The classical scanning electron microscopy was used through the study [65]. The complex electron microscopic approach (TEM + SEM) was employed in the study of *Streptococcus pneumoniae* LocZ mutants morphology and ultrastructure with regard to the cell division process [66]. We showed that Z-ring formation and placement is strongly affected by mutations in the LocZ.

Luckily, we acquired a new SEM in late 2014 opening new laboratory possibilities in sample handling and our own development work. The new FEI Nova NanoSEM 450 is a field-emission gun equipped high resolution **scanning electron microscope** with SE, TLD detectors for secondary electrons, CBS detector for back-scattered electrons and EDAX Octan plus detector for EDS microanalysis.

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Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Biotransformation

Research in the Laboratory of biotransformation integrates chemistry and biology to create frontier areas of molecular biocatalysis, rich in new ideas and results. Novel methodologies, advanced engineered biocatalysts, modern methods of structural analysis and separation procedures generate a number of new compounds that are eventually tested for various biological activities. Extensive collaborations – intramural, national and international – broaden the portfolio of methods and improve the visibility of our research. The Laboratory consists of three informal project subgroups coordinated by senior researchers, *i.e.* "Purple team" (Glycobiology – Křen), "Blue team" (Nitrile biotransformations – Martínková), and "Yellow team" (Flavonoids and antioxidants – Biedermann, Valentová). Coherent collaboration among these teams creates fertile frontier research areas as reflected in the interdisciplinary publications. Fundamental methodology pillars guaranteed by senior experts support the entire research in the group: molecular biology, GMO (Kotik), HPLC (Marhol), LC-MS (Křenková), microbiology and strain collection (Krejzová), technical and material support, and safety (Vávra). Cooperation with industry in the area of ecotechnology (Třinec Iron and Steel Works, CZ; Envisan-Gem, CZ), nutraceuticals (IREL, CZ; Innocrystal, CZ; Bio Base Europe Pilot Plant, BE), food industry (Agra Group, CZ) and specialty chemicals (Phytolab, DE; Carbosynth, UK; Bertin-Pharma, FR) is an important facet of our activities. Strong and long-lasting international collaboration is supported by the number of international (EU FP7, COST) and bilateral (CZ-IT, CZ-DE, CZ-US, CZ-Arg) projects. Solid infrastructure of the group, consisting *i.a.* of a modern instrumentation - HPLC (5 instruments, plus LC-MS - to be installed in Spring 2015), GLC (2x), protein chromatography (3x Äkta), spectrophotometers, shakers, mol. biol. equipment etc. - enables high quality research employing up-to-date methods. Core facilities of the Institute (600 & 700 MHz NMR, FT-MS, el. microscopy) and in the campus (CZ-Openscreen) are indispensable for high quality research.

Major scientific achievements of the group in 2010-2014:

Chemoenzymatic separation of silybin diastereomers. Flavonolignan silybin is a mixture of two diastereomers (silybin A, silybin B) whose separation could only been previously achieved *via* HPLC. We developed chemoenzymatic diastereomeric kinetic resolution of silybin A and silybin B from natural source using Novozym 435 lipase. This method enables the preparation of multigram amounts of pure diastereomers for the investigation of their biological activities and chemical modification. Our partners performed enzymatic screening and we developed the separation process (Monti, Křen et al. *J. Nat. Prod.* 2010, **73,** 613, *IF 3.159*; Gažák et al. *Process Biochem.* 2010, **45,** 1657, *IF 2.444*). We also developed an new separation method of other major silymarin components – silychristin and silydianin using Sephadex LH-20, allowing the first ever isolation of multigram amounts of those compounds

(Křenek et al. *Food Res. Intl.* 2014, **45**, 115, *IF 3.005*). The above chemoenzymatic separation technique allowed us to isolate, so far unknown and by the conventional methods unseparable, *cis*-derivatives of silybin (Novotná et al. *Beilstein J. Org. Chem.* 2014, **10**, 1047, *IF 2.801*) – molecular modelling was accomplished here by Prof. Trouillas (Univ. Limoges, FR) and chiroptical measurements by Dr. Bednárová, Inst. Org. Chem., Biochem, Prague – all remaining work was performed by our group.

Synthesis, antioxidant and antiviral activities of silybin fatty acid conjugates. We have developed two selective acylation methods for silybin esterification with long-chain fatty acids to yield a series of silybin 7-*O*- and 23-*O*-acyl-derivatives with varying acyl chain lengths. The antioxidant and anti-influenza virus activities improved with increasing length of the acyl moiety. This work (Gažák R. et al. *Eur. J. Med. Chem.* 2010, **45,** 1059, *IF 3.269*) was accomplished in cooperation with Palacký University Olomouc and University of Osaka, Japan. Our laboratory designed structures and accomplished the synthetic work and a part of the antioxidant tests.

Synthesis and antiangiogenic activity of galloyl esters of silybin. A new series of galloyl derivatives of silybin were synthesized and extensively tested in a series of antiangiogenic tests with human umbilical vein endothelial cells, resulting in a structure-activity relationship study. 7-*O*-Galloylsilybin B was the strongest antiangiogenic compound with IC_{50} of 4.3 μM. This work (Gažák et al. *J. Med. Chem.* 2011, **54**, 7397, *IF 5.207*) was performed in cooperation with Palacký University Olomouc and University of Málaga, Spain; our group designed all structures and was responsible for the synthetic work.

Chemoenzymatic synthesis of Phase II metabolites. Standards of polyphenolic metabolites (Křen et al. *Curr. Drug Metab.* 2013, **14**, 1009, *IF 4.405*) are essential for the evaluation of their bioavailability (Theodosiou, Křen et al. *Phytochem Rev.* 2014, **13**, 1, *IF 4.333*) and biological activity. We have developed a series of methods for chemoenzymatic synthesis of Phase II phenolic metabolites: Silybin and isosilybin sulfates were prepared by aryl-sulfate sulfotransferase from *Desulfitobacterium hafniense* (Marhol et al., *J. Mol. Cat. B: Enzym.* 2013, **89**, 24, *IF 2.735*), or aryl sulfotransferase IV from rat liver (Purchartová et al. *Appl. Microbiol. Biotechnol.* 2013, **97**, 10391, *IF 3.425*); glucuronides by *Streptomyces* sp. (Charrier, Křen et al. *Mol. Cat. B: Enzym.* 2014, **102**, 167, *IF 2.735*). This work was performed in cooperation with the University of Amsterdam, NL, Aachen University, DE, Bertin-Pharma, FR, Université Paris Descartes, FR, Palacký University Olomouc, and the Institute of Organic Chemistry and Biochemistry, Prague; our group was responsible for the synthetic work, designed the experiments and prepared the manuscripts.

Enzymatic synthesis of isoquercitrin for nutraceutical applications: A novel concept of an "immobilized substrate" was designed (Weignerová et al. *Top. Curr. Chem.* 2010, **295**, 121, *IF 4,291*). New, recombinant α-L-rhamnosidase from *Aspergillus terreus* proved to be thermo- and alkali-tolerant, enabling operation at 70 °C and pH 8.0, which allowed for a high substrate (rutin) load of up to 300 g/L and a high volumetric productivity of the product quercetin-3-β-glucoside. The process has been scaled up to 100 L. (Gerstorferová et al. *Process Biochem.* 2012, **47**, 828, *IF 2.627*; Weignerová et al. *Bioresource Technol.* 2012, **115**, 222, *IF 4.980*; De Winter, Křen et al. *Bioresource Technol.* 2013, **147**, 640, *IF 4.980*; Valentová et al. *Food Chem. Toxicol.* 2014, **68**, 267, *IF 3.010;* Desmet, Bojarová et al. *Chemistry – A Eur. J.* 2012, **18**, 10786, *IF 5.925*; De Winter, Weignerová et al. *Green Chemistry* 2013, **15**, 1949, *IF 6.828*). The major enzymatic part of this work was accomplished by this group, collaborators from Univ. Ghent, BE, contributed within the FP7 project.

New type of substrates and inhibitors for hexosaminidases: New, non-natural substrates for hexosaminidases, based on 4-deoxy hexosamines, were synthesized and tested. These new substances can be also used as substrates for the enzymatic synthesis of glycomimetics (Slámová et al. *Glycobiology* 2010, **20,** 1002, *IF 3.929*; Slámová et al. *Biotechnol. Adv.* 2010, **28**, 682, *IF 8.250*). Based on this new type of substrates, new inhibitors of hexosaminidases were also designed using the multivalency concept (Krejzová et al. *Molecules* 2014, **19**, 3471, *IF 2.428*; Krejzová et al. *Bioorg. Med. Chem. Lett.* 2014, **24**, 5321, *IF 2.331*). Drs. Ettrich and Kulik from Acad. and Univ. Ctr. in Nové Hrady did molecular modelling and Drs. Pelantova and Sedmera measured NMR spectra; all remaining work - structure design, synthesis, biochemistry and paper writing - was accomplished by members of our group.

One-pot enzymatic synthesis of multivalent saccharidic ligands of lectins – was accomplished (2 glycosyltransferases and a UDP-Glc 4'-epimerase) for the first time to yield various poly-LacNAc chains (Rech et al. *Adv. Synth. Catal*. 2011, **353**, 2492, IF 5.250).; these are important ligands for the biofunctionalization of biomaterial surfaces and the construction of an artificial extracellular matrix for tissue engineering (Šimonová et al. *J. Mol. Cat.: B-Enzym.* 2014, **101**, 47, *IF 2.823*; Bojarová et al. *Chem. Soc. Rev.* 2013, **42**, 4774, *IF 28.760*). These are collaborative papers together with the group at RWTH Aachen, D. The contribution of our group comprises enzymatic synthesis, preparation of the oligosaccharide, chemical synthesis of linkers and structure identification as well as submission of the two latter manuscripts.

Enantioconvergent epoxide hydrolases. This work was the first successful *in vitro* evolution of enantioconvergence, an important stereochemical property of epoxide hydrolases (Kotik et al. *J. Biotechnol.* 2011, **156**, 1, *IF 2.970*). All experiments were designed, performed and analyzed by the group members, except for the *in silico* substrate docking experiments performed by Dr. Archelas for better visualization and interpretation of the results. This chiral GC-based methodology was also applied to the *in vitro* evolution of enantioconvergence of a metagenome-derived epoxide hydrolase whose structure has not been experimentally determined, yet (Kotik et al. *J. Mol. Catal. B-Enzym.* 2013, **91**, 44, *IF 2.735*). This work was done in collaboration between our laboratory and the Institut des Sciences Moléculaires de Marseille of the Université d'Aix-Marseille in France.

Metagenomic analysis of microbiome from tetrachloroethene pollution sites. This is the first study describing the total bacterial community of a tetrachloroethene-polluted groundwater site using pyrosequencing of PCR-amplified metagenomic rRNA genes and a subsequent sequence classification procedure (Kotik et al., *Sci. Total Environ.* 2013, **454–5**, 517, *IF 3.286*). This is an intramural collaboration between our laboratory and the Laboratory of Environmental Microbiology.

Nitrilase library. We have characterized over 20 nitrilases from so far unexplored sources, mainly from filamentous fungi (Martínková et al. *Curr. Opin. Chem. Biol.* 2010, **14**, 130, *IF 8.295*). Thus, for the first time nitrilases were reported in genera *Aspergillus*, *Arthroderma*, *Auricularia*, *Gibberella*, *Macrophomina*, *Nectria*, *Neurospora*, *Penicillium*, *Pichia* and *Trichoderma* (Kaplan et al. *Mol. Biotechnol.* 2013, **54**, 996, *IF 2.262*). This is an intramural collaboration between our laboratory and the Laboratory of Molecular Genetics of Bacteria (Dr. Pátek).

Discovery of fungal arylacetonitrilases. Some members of the nitrilase library were recognized to be the first fungal enantioselective nitrilases acting on

arylacetonitriles – „arylacetonitrilases“, which were previously only found in bacteria (Petříčková et al., *Appl. Microbiol. Biotechnol.* 2012, **93**, 1553, *IF 3.425*).

Structure-activity relationships in nitrilases. Site-directed evolution of fungal nitrilases accomplished in collaboration with the University of Stuttgart (Prof. Stolz) within COST action CM0701 refined the knowledge of structure-activity relationships and provided nitrilase variants potentially useful for amide production (Petříčková et al. *J. Mol. Catal. B-Enzym.* 2012, **77**, 74, *IF 2.735*).

New cyanide hydratases and their applications. Cyanide hydratases - related to nitrilases - act preferably on inorganic cyanides. Genome mining resulted in finding the first cyanide hydratases in the genera *Aspergillus*, *Botryotinia*, *Glomerella* and *Penicillium*. The recombinant *E. coli* cells producing cyanide hydratase were used to degrade cyanide in a continuous way in collaboration with Univ. L´Aquila (Prof. Cantarella; COST Action CM0701). We were also the first to report on the activities of cyanide hydratases for nitriles such as fumaronitrile and 2-cyanopyridine (Rinágelová et al. *Proc. Biochem.* 2014, **49**, 445, *IF 2.414*). The purified cyanide hydratase was used to develop a novel method for the determination of cyanide in wastewaters, submitted for validation to the Ministry of Environment of the Czech Republic.

Nitrile-transforming enzymes in cascade processes. Nitrile hydratases were used for the production of hydroxamic acids, chelating agents with pharmaceutical applications (Vejvoda et al. *J. Mol. Catal. B-Enzym.* 2011, **71**, 51, *IF 3.330*). The nitrile hydratase-amidase systems developed by us were also employed in multistep chemoenzymatic procedures designed by CNR Catania, IT to prepare new cyclitols as building blocks of natural compounds (D´Antona et al., *Tetrahedron: Asymmetry* 2010, **21**, 695, *F 2.625*; D´Antona et al. *Tetrahedron: Asymmetry* 2010, **21**, 2448, *IF 2.625*). Other applications of nitrile hydratase-amidase systems comprise a mild hydrolysis of sensitive substrates such as heterocyclic nitriles (Cantarella et al. *Enzyme Microb. Technol.* 2011, **48**, 345, *F 2.287*; Cantarella et al. *Proc. Biochem.* 2012, **47**, 1192, *F 2.627*) and cyanohydrins (Rucká et al. *A. van Leeuwenhoek* 2014, **105**, 1179, *F 2.072*) into respective carboxylic acids.

Nitrile-transforming enzymes in herbicide biodegradation. The ability of the nitrile hydratase-amidase system in rhodococci (common soil microorganisms) to degrade benzonitrile herbicides (bromoxynil, ioxynil) was shown for the first time. The metabolites (amides, acids) were isolated and identified by us and examined for toxicity at the Inst. Chem. Technol. in Prague. Since the toxicity of the metabolites was only slightly reduced as compared to the parent compounds, a complete detoxification would require further steps (Veselá et al. *Biodegradation* 2010, **21**, 761, *F 1.783*).

Biodegradation of phenolic compounds. We have shown that laccase is a potent tool for the degradation of tetrabromobisphenol A (TBBPA, flame retardant). In collaboration with BOKU Wien (Dr. Ludwig, Prof. Haltrich; COST CM0701) we also found that cellobiose dehydrogenase acts synergistically in the degradation of TBBPA by laccase. We were the first to identify the products of TBBPA transformation by laccase and found them to be analogous to mammalian metabolites, this opened the way for the production of these metabolites in high yields for toxicology studies (Uhnáková et al. *Bioresource Technol.* 2011, **102**, 9409, *IF 4.365*).¨

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Enzyme Technology

Multidisciplinary, research laboratory focused on oriented research in microbiology, biotechnology, protein engineering, bioinformatics and biocatalysis. Principle outcomes of the laboratory: research papers, patents, development and transfer of bioprocesses, contract research, student supervising. The laboratory success in oriented research could be documented by high percentage of patent transfer into practice: six from ten patents granted during the evaluated period were adopted by industry.

Ongoing projects of the laboratory (basic and oriented research)

Metagenom from polluted environment as a source of novel prokaryotic genes.

Targeted metagenomics was successfully used to mine structural genes encoding environmental epoxide hydrolases, Baeyer-Villiger monooxygenases, penicillin acylases and α-amino acid ester hydrolases. To increase probability of positive outcomes, environmental samples from polluted area were always involved (e.g., polluted pharmaceutical factory area). Gene-mining was performed using homology gene-specific degenerate primer PCR, semi-specific genome walking PCR and standard PCR on metagenomic DNA templates and complete genes were cloned and expressed in *Escherichia coli* host strains.

This way, enantioselective and enantioconvergent recombinant epoxide hydrolase Kau2 has been obtained from biofilter-derived eDNA (Kotik et al., J Mol Catal B-Enzym 65, 2010; Grulich et al., Process Biochem 46, 2011). The enzyme produced by recombinant culture in CSTR under optimized conditions was characterized, immobilized on oxirane-activated supports and used in conversion of aromatic epoxides such as styrene oxide, p-nitrostyrene oxide, and trans-1-phenyl-1,2-epoxypropane.

The environmental genes encoding penicillin acylases and α-amino acid ester hydrolases are currently used in projects dealing with molecular evolution of enzymes. The gene mining is performed with eDNA from polluted soil samples from surroundings of pharmaceutical factories producing beta-lactams or cultivable microorganisms of these soils XXX.

Heterologous expression of novel or synthetic genes, directed evolution of enzymes and construction of chimaeric proteins. Development of high-expression systems and their evaluation (bacterial or yeast hosts)

Constitutive or inducible high-expression systems based on bacterial or yeast hosts were developed and their synthetic potential was evaluated in continuously stirred bioreactor cultures: s-*npga* of *Achromobacter* sp. in *Pichia pastoris* (industrial partner), *aeh* gene from *B. cereus* in *E. coli* (a new nature isolate and producer of AEH; Diploma project of A. Pitkina), *aeh* gene from *Stenotrophomonas maltophilia* in *E. coli* (the first report on cloning, Diploma project of J. Zahradník), *aeh* gene encoding chimaeric AEH in *E. coli* (improved AEH; patent application), leader-less *pga* gene of *E. coli* in *P. pastoris* (cytosolic expression of extracellular enzyme, Marešová et al., *BMC Biotechnology 10,* 2010), cloning of gene encoding morphinone reductase of *Agrobacterium radiobacter* in *E. coli* (novel psychrophylic

enzyme, J. Zahradník, Diploma project), m-*npga* of *Achromobacter* sp. in *E. coli* (mutant with AA replacement in beta-subunit, M. Grulich, PhD thesis).

Research into enantioselectivity of enzymes and whole-cell catalysts: production of chiral compounds (APIs) by biocatalysis

Goal of oriented research in cooperation with pharmaceutical company was to exploit biodiversity of soil microbial consortia for designing "green chemistry" processes for production of APIs based on pure enantiomers. The strain *Rhodococcus fascians* was isolated that catalyzed production of Ezetimibe from ketone precursors with high degree of conversion and *de* of 99.9% (Kyslíková et al., J.Mol.Catal.B: Enz 67, 2010). Esomeprazol was efficiently prepared using novel strain *Lysinibacillus* sp. B71 CCM 7718 biotransforming prochiral sulfide into (S)-omeprazol without consequent oxidation into sulfone with *ee*(S) 100% (Babiak et al., Bioresource Technology 102, 2011; Babiak et al., CZ patent 302612, 2011). The performance of both whole-cell catalysts is unique and was optimized at laboratory scale including cofactor regeneration. We have published a pilot publication describing for the first time hydroxylation of codeine and morphine at C-14 by gram negative bacterium classified as *Rhizobium radiobacter* R89-1 CCM 7947 (Kyslíková et al., J. Mol. Catal. B: Enz 87, 2013). The strain is tolerant to high concentrations of morphine alkaloids which makes it good candidate for development of catalysts suitable for replacements of chemical reactions in syntheses of APIs based on morphine alkaloids by biocatalysis. Two granted national patents and PCT application came out of the project (Babiak et al., CZ patent 303607, 2012; Babiak et al., CZ patent 304116, 2013; Babiak et al., WO 2013075676 A1).

Taxonomical classification of nature isolates (cultivable microorganisms) and members of microbial consortia (eDNA)

To cover several ongoing laboratory projects, techniques required for taxonomical classification of nature isolates (prokaryotes) and members of microbial consortia from eDNA were adopted. In context with this laboratory skill XXX, cooperation on taxonomical classification of ascomycetes between the Institute of Microbiology CAS and the Institute of Botany CAS was built up. Based on morphological data and phylogenetic analysis of nucleotide sequences of small and large subunits of nuclear ribosomal DNA and structural gene encoding the second largest subunit of RNA polymerase II, several new genera and species were described. Relationships among five morphologically similar strains designated as *Exochalara longissima* were resolved and it was found that these strains belong to three different taxonomic genera - two of them (*Brachyalara* and *Infundichalara*) were newly identified and described (Réblová et al., *Fungal Diversity* 46, 2011). Another studies of representatives of class *Sordariomycetes* resulted in description of new genera (*Pleurotheciella, Xylochrysis*) and species of ascomycetes (Réblová et al., Mycologia 104, 2012, Réblová et al., Mycologia 106, 2014).

Microbial secondary products: siderophores of bacteria and basidiomycetes

The project dealt with elementary research of physiology of pathogen microorganisms. Goal of work was to construct a mutant strain of *Pseudomonas aeruginosa* bearing modified regulatory Fur protein (Valešová et al., Folia Microbiologica 58, 2013). The research was focused on a study of the correlation between the production of siderophores production of which is considered as a factor of pathogenicity, and their specific outer membrane receptors (Junior researchers grant KJB500200703). Regulatory mutant overproduces siderophores and specific receptor for pyochelin at presence of FeIII ions and can be used as a test microorganism for efficiency of novel, targeted compounds such as conjugates antibiotic-

siderophore (Palyzová et al., CZ patent 301848, 2010) or deregulated production of siderophores may be applied to control the growth of phytopathogens (oriented research started in 2015).

„In silico"-based prediction of biocatalytic steps: replacement of chemistry by biocatalysis

Bioinformatics and "in silico" experiments (molecular modelling of structures of penicillin acylases, docking experiments, resolution of protein crystal structures) make it possible to identify and select the substrates convenient for enzymatic steps in bioprocesses for production of APIs or beta-lactams. Analogously, homologous models of enzyme mutants may be used in *in silico* experiments without need to construct recombinant expression strains *in vivo*. Large experimenal enantioselective study with PGA (PGA^A of *Achromobacter* sp.) was performed using selected alpha/beta amino acids racemates to get optically pure compounds useful as building blocks of active pharmaceutical ingrediences (APIs). The PGA showed higher stereoselectivity for three enantiomers of N-phenylacetylated-β-homoleucine, α-*tert*-leucine and β-leucine. To study the mechanism of enantiodiscrimination on molecular level, we have constructed a homology model of PGA^A and performed analogous enantioselective study by molecular docking experiments. *In silico* experiments confirmed the data from enzymatic resolutions and validated the model structure (Grulich et al., Applied Microbiol. Biotechnol., 2015 submitted). This approach was used to predict high enantiopreference of PGA^A towards five novel substrates with application potential. Recently, 3D-structure determination of PGA from *Achromobacter* sp. by crystallization experiments has been started (M. Grulich, Doctoral dissertation thesis).

As a result of a long-term research into penicillin acylases, a pioneering review on industrial potential of PGA enantioselectivity and promiscuity was published (Grulich et al., Biotechnology Adv. 31, 2013). The paper represents the first review covering PGA-catalyzed reactions other than biotransformations of β–lactams.

Development of penicillin acylase catalysts for kinetically-controlled synthesis of beta-lactam antibiotics (enzyme characterization, immobilization and catalyst characterization)

The project is a logical continuation of multidisciplinary, long-term research dealing with biotransformation of beta-lactams and was carried out in cooperation with pharmaceutical industry. A company developed the way of immobilization (catalyst Fermase NA™ 150), the laboratory developed the expression system, optimized and scaled-up fermentation process and DSP, developed the way of CLEA preparation, and characterized final catalyst and conversion process. The technology was transferred into industry in 2011 XXX Six national and international patents (IN, 2 US, EP, 2 CN) were granted since 2010. Publication dealing with kinetically controlled syntheses of β–lactam antibiotics (Bečka et al., Appl. Microbiol. Biotechnol. 98, 2014) is a scientific outcome of the latest stage of this R&D process. The novel acylase of soil microorganism *Achromobacter* sp. was superior to that of *E. coli* for syntheses of Ampicillin and Amoxicillin under industrial conditions due to its better parameter S/H, higher accumulation of the product in whole range of reactant concentrations and conversion degree. Immobilized NPGA exhibited long operation stability with half-life of about 2000 cycles for synthesis of amoxicillin at conversion conditions used in large-scale processes.

Contract research – Design and development of pilot-scale bioprocesses

Production of haloalkane dehalogenases in a stirred bioreactor

The research was aimed at development of the fermentation process for production of the haloalkane dehalogenase LinB (an enzyme used as a biosensor) in continuously-stirred tank reactor (CSTR) at pilot scale of 180 l (working volume), further scale-up to 1000 l and large-scale down-stream process yielding enzyme preparations of different purity. During the process development, medium composition and culture conditions in CSTR for enzyme production were optimized at laboratory scale of 6 l (a fed-batch culture) and further scale-up of up-stream and down-stream processes was performed in cooperation with Biotechnology hall, a facility of Institute of Microbiology XXX.

Development of an inducible, high-expression system for α-amino acid ester hydrolase

Using gene-specific degenerate primers, semi-specific genome walking PCR and standard PCR on chromosomal DNA template, the gene encoding α-amino acid ester hydrolase from *Sphingomonas* sp. (provided by Fermenta Biotech Ltd., India) was obtained, cloned in *E. coli* and a high-expression bacterial systems based on pET expression vector was constructed. The way of fed-batch cultivation and induction regime for expression system was optimized and scaled-up in CSTR to the working volume of 6 l. The process was transferred to pharmaceutical company (Kyslíková et al., Transfer of technology for production of AEH from *Sphingomonas* sp., 2014)

3. Research Plan of the team for 2015–2019

Maximum length of 3 pages.

Tight cooperation between basic research and oriented research groups should yield laboratory outcomes in the following areas:

Microorganisms with novel metabolic capabilities for bioremediation and biosensing of pharmaceuticals, xenobiotics presenting serious threat to environment

Finding and characterization of biotransformation potential of a nature microorganisms from contaminated areas capable of degradation of pharmaceutical residua. Promising isolates will be characterized phenotypically, taxonomically and used in process of xenobiotic bioremediation or utilization. Enzymes/metabolites involved in APIs metabolism will be identified and used to develop sensors for xenobiotics biosensing (APIs to be studied were recently added or are expected to appear soon on watch-lists: Diclofenac, Sulfamethoxazole, morphine alkaloids).

Biocontrol strategies against phyto-pathogens

The research is focused on siderophores playing a significant role in the biological control mechanism against certain phyto-pathogen (*Fusarium oxysporum, Xanthomonas campestris* pv. *campestris*). Project will detect and quantify pathogens in both the seed and the soil before planting. Siderophores of pseudomonads (pyoverdine, pyochelin) will be applied as different formulations. The project is implemented into project supported by Ministry of Agriculture (NAZV) in 2015-2018.

Microbial ecosystems affected by anthropogenic activity: targeted metagenomics and synthetic biology approaches

Research into prokaryotic consortia aimed at relationship between anthropogenic activity and biotransforming potential of the consortium. Function-based screening of total environmental DNA and sequence-based screening of PCR-amplicons performed on the environmental DNA template will be used as tools for evaluation of a microbioconsortium as to decomposition potential for specific pollutant (e.g., Diclofenac, Sulfamethoxazole, Codeine derivatives, Triclosan, and Triclocarban). Consequently, sophisticated expression systems for xenobiotic complete utilization will be developed *via* synthetic biology approach (molecular evolution of wild type genes and construction of artificial biotransformation pathways).

Bioinformatics and enzyme structure determination

3D-structures of novel penicillin acylases will be determined by crystallization studies or by electron cryoscopy. Structure determination will be complemented with *in silico* homology models. For predictions of penicillin acylases applications in kinetically controlled syntheses of beta-lactam antibiotics, advanced techniques such as molecular dynamics and "covalent-docking" experiment with two substrates will be applied in docking experiments.

Development of process for large-scale production of catalyst based on Alpha-amino acid ester hydrolase

AEH is an enzyme with high biocatalytic potential. For instance, as to synthesis of beta-lactam antibiotics the enzyme offers several advantages over PGA. However, any wild-type AEH requires improvement of enzyme traits. Engineered AEH from *Achromobacter* sp. CCM 4824 convenient for large-scale synthetic process (parameters: S/H ratio, wide temperature range for synthetic activity optimum) will be prepared via functional screening among mutant and chimaeric *aeh* genes. Production of the enzyme by high expression system and DSP will be optimized and scaled-up. Robust catalyst will be designed and biocatalytic potential evaluated.

Exploitation of laboratory expertise to evaluation of application potential of basic research outcomes at IM

Expertise of laboratory staff and available equipment/methodology make the laboratory qualified and specialized facility to experimental evaluation of application potential of basic research results (generated elsewhere on IM or Academic campus Krč). Currently, the laboratory is the only unit at IM capable of these activities including scale up of processes up to pilot-experiment scale and, thus, initiate the process of technology transfer. The laboratory bridges the gap between the laboratory generating results with application potential and a potential customer requiring pilot-plant experiment data (the latter being acquired in cooperation between the Laboratory of Enzyme Technology and Biotechnological Hall, IM). The laboratory might offer evaluation of applicable potential for most of outcomes in the field of microbiology/biotechnology.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Cell Reproduction

Laboratory of Cell Reproduction has a long tradition working with yeast *S.cerevisiae* as model system of eukaryotic cells. We employed a routine use a set of modern techniques of molecular and cell biology namely live-cell fluorescence microscopy. Whereas the composition of the team has changed during the evaluated period (several maternity leaves Vasicova, Frydlova and Janoskova; or retirement leaves of Streiblova and Opekarova), a great advantage of the laboratory are still well-trained and experienced specialists in all the technical fields needed – microscopy, molecular genetics, protein analyses (including integral plasma membrane proteins) and the essential laboratory equipment suitable for such a combined methodology.

The main topics of the laboratory in the evaluated period included i) stress-induced protein accumulations, ii) protein import into the nucleus and iii) protein redistribution into the plasma membrane domains.

Composition of heat shock stress granules

During the evaluated period we continued in analyses of heat induced stress granules (SGs). Besides translation initiation factors, we identified also **translation elongation factors eEF3 (Yef3p)** and **eEF1B2 (Tef4p)** as well as **translation termination factors eRF1 (Sup45p)** and **eRF3 (Sup35p)** as new components of SGs. We proved that the **"prion"** domain of eRF3/Sup35 has no obvious role in heat-induced SGs assembly (Grousl et al., 2013, *PloS one*. 8:e57083).

We also found that with heat-induced SGs also associates protein **Mmi1, *S. cerevisiae* homolog** of the **human translationally controlled tumor protein TCTP.** In collaboration with M. Breitenbach's laboratory (Univ. Salzburg), we also discovered that also accumulates in the nucleus upon robust heat shock. We found that ***mmi1*Δ** mutant strains display **increased** proteolytic activity of the **proteasome** and they are **resistant** to robust heat shock (Rinnerthaler et al., 2013, *PloS one*. 8:e77791). Consistently, our detailed microscopic analyses confirmed our predictions that Mmi1 affects distribution of phosphatase Cdc14.

Signaling patways affecting formations of heat shock stress granules

In searching for the signaling pathway, we also continued in phenotypic analyses of the ts mutant *rpg1-3* of the essential gene *RPG1*/*TIF32*/eIF3a. Our data indicate that inhibition of translation (either initiation or elongation) results in formation of "SGs seeds" depending on mRNAs from ongoing translation. Upon persisting stress, these "SGs seeds" reorganize into large SGs and associate with the markers of misfolded proteins (the manuscript on this topic is in preparation).

We also identified two mutant strains defective in heat-induced SGs formations. In collaboration with S. Valentini (Brasil) we discovered that the A3 ts mutation of the translation factor **eIF5A** strongly affects assembly of heat-induced SGs, recovery of the actin cytoskeleton and stress survival. eIF5A is responsible for translation of prolin-rich proteins.

Our experiments revealed that also deletion of **Eap1**, a prolin-rich protein involved in regulation of translation initiation, also affects formation of heat-induced SGs. These data point to the role of prolin-rich proteins in stress response.

Software for evaluation of proteins granularity

For evaluation of protein granularity we used the new software tool – oCellaris, which has been recently developed in our laboratory in collaboration with the Czech company DEL (http://www.ocellaris.cz/index.php/en/) (the manuscript is in preparation).

Proteostasis in yeast cells after oxidative stress, heat stress and in post-diauxic cells

Since proteostasis is strongly affected by reactive oxygen species (ROS), we identified and characterized (in collaboration with M. Breitenbach's laboratory; Univ. Salzburg) protein **Yno1/Aim14** as a genuine **NADPH oxidase** of *S.cerevisiae*. Several independent lines of evidence point to regulation of the actin cytoskeleton by ROS produced by Yno1p (Rinnerthaler et al., 2012, *Proceedings of the National Academy of Sciences of the United States of America*. 109:8658-8663).

Using live-cell imaging, we also documented that actin bodies in yeast post-diauxic cells are not markers of yeast quiescence or replicative age of individual cells but more likely these actin structures are connected to **mitochondrial dysfunction** (manuscript Vasicova et al., J. Cell Biology, prepared for submission).

In collaboration with J. Malinsky from IEM ASCR, we showed that the main exoribonuclease **Xrn1/Kem1** is redistributed from cytosol to the plasma membrane compartment **MCC** (**M**embrane **C**ompartment of **C**an1) in early **post-diauxic** cells, but even this Xrn1 can still associate with heat-induced stress granules (Grousl et al., 2015, *PloS one*. 10:e0122770).

We used specific microscopic analyses to localize proteins in different nuclear processes.

Our new tagging system was used to localize Vps15 and Vps34 proteins (both form membrane-asociated signal transduction complex) within the nucleus-vacuolar junctions where they facilitate transcription of genes positioned at the nuclear pheriphery. We proved the **new concept of regulation of transcription (**Gaur et al., 2013, *Genetics*. 193:829-851). To understand the import of proteins into the nucleus, we also performed detailed analyses of imitation-switch chromatin-remodeling factor **Isw1** (deletion and direct mutagenesis) in respect to its nuclear localization signal. Our data proved that **Isw1** contains bipartite NLS with non-conventionally long linker. We confirmed that its nuclear import is mediated by the classical import pathway (Vasicova et al., 2012, *Traffic*. 14:176-193).

Based on the original data on compartmentalization of plasma membrane proteins we also continued in collaborations with J. Malinsky (IEM ASCR) and W. Tanner (Univ. Regensburg) in studies of **the MC compartment** (Loibl et al. 2010, *Eukaryotic Cell* 9, 1184-1192). We showed that the function of protein **Nce102** (non-classical export) is to direct the specific permeases into MCC. Our experience with this topic has been recognized in the field also by invitation to several well accepted reviews (Malinsky et al., 2013, ***Annu. Rev. Plant. Biol.* 64, 501-529.** doi:10.1146/annurev-arplant-050312-120103; Tanner et al., 2011, *Plant Cell* 23, 1191-1193, Opekarova et al., 2010, *Plant Biology* 12, 94-98).

Tradition and experiences

Whereas the experimental research of the founding member of the laboratory, Professor emeritus Eva Streiblova, has been focused on a completely different fungus ***Tuber,*** *s.p*. during the last few years in the institute (Streiblova et al., 2012, *FEMS Microbiol Ecol*. 80:1-8), her advice related to yeast cell biology was always very helpful to all members of the laboratory.

So far, the laboratory has always found the main funding by the subsequent grants from CSF agency (2009-2011, 2012-2015), as well as one grant from the technology agency (2010-2011). The topics are still attractive for undergraduate and graduate students. Because of funding limits we had to refuse several student applications. Also international collaborations were always supported by small bilateral projects. Because of organization of highly appreciated and successful conference in 2001, Jiri Hasek has been offered by the yeast community (Professor Hohmann, Chair of the Finance and Policy Committee) to organize also the 28th International Conference on Yeast Genetics and Molecular Biology in 2017.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Genetics of Bacteria

Research in the Laboratory of Molecular Genetics of Bacteria has been devoted to (1) regulatory mechanisms of transcription in Gram-positive bacteria, (2) biodegradations, bioremediations and biotransformations, (3) antibacterial activities of novel compounds and (4) restriction-modification systems of Type I.

(1) Regulatory mechanisms of transcription in Gram-positive bacteria,

(a) Transcription in *Corynebacterium glutamicum*

Our research activities have been for a long time focused on the genetics of *Corynebacterium glutamicum*, an organism which is widely used in biotechnology and which has also become a new model organism for corynebacteria and other actinobacteria. In the past, we analyzed particularly promoters of housekeeping genes and used this knowledge e.g. for the development of valine producing strains. Within the years 2010-2014, our interest shifted to the genes controlled by alternative sigma factors (which are mostly responsible for various stress responses) and structures of the respective promoters. To assign sigma factors to the individual promoters and to study other transcriptional features in *C. glutamicum*, we have developed several new techniques.

Development of the unique *in vitro* transcription system for *Corynebacterium glutamicum*

This system consists of *C. glutamicum* RNA polymerase (RNAP) core enzyme and sigma subunits expressed and purified separately. The reconstituted *C. glutamicum* RNAP holoenzyme was used to define sigma factor dependency of the genes and their particular promoters. The system can be further used to analyze transcriptional control by various regulatory proteins in *C. glutamicum* (Holátko et al. Appl. Microbiol. Biotechnol. 96:521-529, 2012).

Two-plasmid system for assignment of sigma factors to promoters in *C. glutamicum*

We developed the system for the analysis of function of alternative sigma factors. It consists of an expression vector with cloned sigma factor genes and promoter-test vector with the analyzed promoter and the *gfp* reporter gene. Using the combination of the *in vitro* and *in vivo* techniques we are able to reliably define which sigma factor recognizes the individual promoter. We have already discovered promoters of *C. glutamicum* dependent on SigE and SigD for the first time.

Transcriptional control of the operon encoding sigma factor SigH and its anti-sigma factor RshA

In cooperation with the group from Bielefeld University (Germany) we described the regulatory mechanisms, which control transcription of *sigH* and *rshA* genes and revealed genes and promoters controlled by SigH (Busche et al. BMC Genomics 13:445, 2012). SigH-dependent regulatory network in *C. glutamicum* was proposed. We participated in this study by the determination of a number of the SigH-dependent promoters and their analysis.

(b) RNA polymerase of *Bacillus subtilis* and mycobacteria

In 2010-2014, we significantly contributed to the knowledge of regulatory molecules (proteins, RNA and DNA sequences) interacting with RNAP from *Bacillus subtilis* and *Mycobacterium smegmatis*. We

discovered and/or characterized subunits or binding partners of RNAP from *Bacillus subtilis* (promoter DNA, delta and epsilon subunits, HelD, YdeB, GreA) and *Mycobacterium smegmatis* (Ms1).

Characterization of the delta subunit of RNA polymerase of *B. subtilis*

We characterized the delta subunit of RNAP from *B. subtilis*. We demonstrated that this subunit potentiates RNAP for regulation by the concentration of the initiating nucleoside triphosphate (iNTP). This is important for rapid changes in gene expression in response to nutritional changes in the environment and also for virulence of pathogenic species. We showed that two other proteins previously thought to implicate in RNAP regulation by [iNTP], GreA and YdeB, have no role in this process (Rabatinova et al., J. Bacteriol. 195:2603-2611, 2013). In collaboration with Masaryk University, Brno and University of Warsaw, we solved the 3D structure of the delta subunit (Motackova et al. Proteins 78:1807-1810, 2010; Papouskova et al, Chembiochem. 14:1772-1779, 2013; Demo et al., J. Struct. Biol. 187:174-186, 2014) and contributed to a number of methodical NMR papers (e. g. Kaderavek et al. J. Biomol. NMR 58:193-207, 2014; Zawadzka-Kazimierczuk et al., J. Biomol. NMR 52:329-337, 2012; Novace et al. J Biomol. NMR 50:1-11, 2011).

DNA elements required for rapid changes in gene expression in *B. subtilis*

Ribosomal RNA (rRNA) promoters in *B. subtilis* are regulated by the concentration of the iNTP, namely GTP. This regulation in *Escherichia coli* is dependent on the promoter sequence. However, the rules derived from *E. coli* are not applicable to *B. subtilis*. Hence, we identified the 3´region of *B. subtilis* rRNA promoters that corresponds to the transcription bubble as the key sequence that determines this regulation. Within this region, we identified -5T as an important base required for this regulation. This study significantly contributed to understanding of the transcription machinery in Gram-positive bacteria (Sojka et al. Nucleic Acids Res. 39:4598-611, 2011).

The epsilon subunit of RNA polymerase

In collaboration with an Australian group from Newcastle, led by Peter Lewis, and our French collaborators (O. Delumeau, Paris), we proposed that YkzG protein is a subunit of RNAP in *B. subtilis*. We prepared a YkzG-null strain, characterized this protein by *in vitro* transcription and proposed that it may function as an immunity protein, mimicking a bacteriophage Gp2 protein that inhibits RNAP in *E. coli*.

The YkzG protein was formerly known as omega I, whereas the omega subunit of RNAP common to all bacteria is designated omega II. To avoid confusion we suggested a new designation of the YkzG protein (epsilon subunit of RNAP) and a new name for the respective gene (*rpoY*) (Keller et al. J Bacteriol. 196:3622-3632, 2014).

Spx, a pleiotropic transcription regulator

The genome-wide effect of the transcription factor Spx was determined in *B. subtilis*. This regulator is important for maintaining the redox homeostasis. Our French collaborators conducted ChIP-on-chip experiments with tagged Spx and we prepared a defined cell-free transcription system where we tested the regulatory properties of Spx on selected promoters. This study thus globally characterized the Spx regulatory network, revealing its role in the basal expression of some genes and its complex interplay with other stress responses (Rochat et al. Nucleic Acids Res. 40:9571-9583, 2012).

HelD, a newly discovered interaction partner of RNAP

We discovered a new interaction partner of *B. subtilis* RNAP, HelD (Wiedermannova et al. Nucleic Acids Res. 42:5151-5163, 2014). We showed that this helicase-like protein binds to RNAP in the vicinity of the secondary channel and stimulates transcription. This effect is augmented by the delta subunit. The stimulation is mechanistically mediated by enhancing recycling of RNAP, i.e. by allowing more rounds of transcription. Our Australian collaborators participated on the identification of the binding site of HelD on RNAP.

Discovery of Ms1, a small RNA interacting with RNAP in mycobacteria

In collaboration with the Laboratory of Bionformatics of the Institute of Microbiology (J. Vohradský), we discovered a highly abundant small RNA in *Mycobacterium smegmatis*, which was designated Ms1. The colleagues carried out the bioinformatics analysis while we provided the experimental evidence (Panek et al., Nucleic Acids Res. 39:3418–3426, 2011). Subsequently, we identified RNAP as the cellular binding partner of Ms1 (Hnilicova et al. Nucleic Acids Res. 42:11763-11776, 2014). Importantly, we showed that Ms1 binds to the core RNAP. This is in contrast to the previously described 6S RNA, that interacts with complete RNAP holoenzyme. We further investigate the function of Ms1.

(2) Biodegradations, biotransformations and bioremediations

Studies of biodegradation of toxic compounds in the environment concentrated in 2010-2014 to organic pollutants such as phenol, petroleum hydrocarbons, polychlorinated biphenyls, chloroethenes and nitriles, mechanisms of their biodegradation, involved enzymes and metagenomic identification of the involved genes within the respective microbial communities.

(a) Degradation of aromatic compounds in *Rhodococcus* strains

Strains of the genus *Rhodococcus* are also bacteria of our long-term interest due to their broad abilities to degrade various organic substances, particularly toxic compounds contaminating the environment. We concentrated on the degradation of aromatic compounds by *Rhodococcus erythropolis* and *R. jostii* within the AROMAGEN project, which also included two university groups and two environmental companies. Clusters of genes responsible for phenol and catechol catabolism were identified and characterized in the two *Rhodococcus* strains. We described transcriptional regulation of the respective genes.

Mechanisms of phenol degradation genes in *Rhodococcus* strains

The major result of the genetic studies of mechanisms controlling expression of genes responsible for phenol degradation was the description of induction and carbon catabolite repression of phenol degradation genes in *R. erythropolis* and *R. jostii* (Szőköl et al. Appl. Microbiol. Biotechnol 98:8267-8279, 2014).

Construction of phenol-degrading *Rhodococcus* strains

Knowledge of the genetic determination of phenol catabolism and the respective regulatory mechanisms enabled us to construct several strains which degraded phenol more intensively than the parental strain. These strains were applied for both degradation of phenol in model media and removal of phenol from real wastewater (Zídková et al., Int. Biodeter. Biodegr. 84:179-184, 2013).

Determination of the complete genome sequence of *Rhodococcus erythropolis* CCM2595

Our studies showed that the used *R. erythropolis* strain provides both wide biodegradation capacities and convenient biotechnological properties, such as biofilm formation. In cooperation with the group from Institute of Molecular Genetics ASCR, the complete genome sequence of *Rhodococcus erythropolis* CCM2595 was determined (Strnad et al., Genome Announc. 2: e00208-14, 2014). It was only the fifth complete genome of a *Rhodococcus* strain available in the GenBank database. Our smaller contribution to this project consisted of preparation of DNA material for sequencing, description of the strain and assistance in genome annotation.

(b) Metabolism of nitriles, characterization of the genes and enzymes

Our continuous collaboration with the Laboratory of Biotransformations of the Institute of Microbiology resulted in a number of studies describing genes encoding the industrially useful enzymes in bacteria, fungi and plants and unique activities of the corresponding enzymes. In the last years we focused on

the genes encoding enzymes of nitrile metabolism. Our efforts in detection, isolation and cloning of the genes and genetic analyses of their sources are complementary to the enzymatic analyses done in the Laboratory of Biotransformations.

Degradation of benzonitrile herbicides

Benzonitrile herbicides (e.g. chloroxynil and dichlobenil) are slowly mineralized in soils. We found the ability of *Rhodococcus erythropolis* and *R. rhodochrous* strains to degrade some of these herbicides and decrease their toxicity. Our task in this study was to isolate and clone the genes encoding the respective enzymes (nitrile hydratases) catalyzing the conversion of the herbicides. We also used the bacterial strains to test the herbicide degradation. (Veselá et al. J. Ind. Microbiol. Biotechnol. 39:1811–1819, 2012).

Expression control of nitrile hydratase and amidase genes in *Rhodococcus erythropolis*

We described constitutive and induced expression of the genes encoding enzymes of nitrile metabolism in *R. erythropolis* strains. Moreover the substrate specificities of the enzymes were tested. *R. erythropolis* nitrile hydratase and amidase were found to be efficient at hydrolyzing cyanohydrins which are precursors of industrially useful amides and hydroxyl acids, such as (R)-2-chloromandelic acid used as a building block for synthesis of an antiplatelet agent and acetylcholine esterase inhibitors (Rucká L. et al., Antonie van Leeuwenhoek 105:1179–1190, 2014).

A comparative study of nitrilases identified by genome mining

A number of genes encoding bacterial and fungal nitrilases and cyanide hydratases were found by data mining, synthesized and expressed. The produced enzymes were purified and analyzed. Promising nitrilases for the transformation of industrially important substrates were found, e.g. the nitrilase from *Rhodococcus ruber* converting cyanopyridines and bromoxynil and cyanide hydratase from *Aspergillus niger* degrading KCN (Kaplan et al., Mol. Biotechnol. 54:996-1003, 2013). We contributed to the study by cloning and expression of the genes and purification of the enzymes.

(c) Microbial communities at the polluted sites and remediation technology

Our research activities have also been focused on genetic analysis of bacterial aquifer communities degrading xenobiotics (petroleum hydrocarbons, polychlorinated biphenyls, and chloroethenes), assessment and prediction of natural attenuation processes in contaminated soil and groundwater and analysis of the metagenomic organization of genes with functional priority for degradation of aromatic hydrocarbons in highly contaminated environments. Bioremediation was carried out using the plant-microbial systems for degradation of organic soil contaminants.

Adaptability, functionality and diversity of microbial communities under the strong selective pressure exerted by post-remediation pollutant reversal

We have carried out a pioneer study on the post-remediation pollutant reversal and its strong selective pressure on diversity, adaptability and biodegradation functionality of microbial communities. High petroleum hydrocarbon concentration in mesocosm systems led to selection of phylogenetically diverse microbial populations with increased natural attenuation and phytoremediation performance (Korotkevych et al., FEMS Microbiol. Ecol. 78: 137-149, 2011).

Methods for detection of diffuse pollution in environmental samples

Diffuse pollution is a long-term contamination of the environment by very low concentration of pollutants released or spilled due to variety of human activities. It leads to negative environmental impact including eutrophication, loss of biodiversity, and harm to human health. We developed new methods for multi-criteria assessment of environmental samples contaminated with aromatic compounds. An absolute quantitative real-time polymerase chain reaction method (qPCR) was developed for quantification of a diverse group of catabolic genes with relevance to the microbial

degradation (Brennerová, M. and Stavělová, M., Methodology for detection of diffuse pollution in environmental samples. ID: RIV/61388971 - Functional model, 2011; Brennerova et al., Microbes in Applied research: Current Advances and Challenges. pp. 157-161, 2012).

(3) Activities of antibacterial compounds

Discovery of new antibacterial compounds, lipophosphonoxins (LPPOs)

Lipophosphonoxins displayed significant antibacterial properties against a panel of Gram-positive species, including multiresistant strains. The minimum inhibitory concentration values of the best inhibitors are in the 1−12 μg/mL range, while their cytotoxic concentrations against human cell lines are significantly above this range (Rejman et al. J. Med. Chem. 54:7884-7898, 2011). These compounds are promising new antibiotics. The work was done in collaboration with the Institute of Organic Chemistry and Biochemistry, Prague. We contributed to the study by participating in planning the project, testing the effects of the compounds on selected bacterial strains and conducting the tests of LPPOs on enzymes *in vivo*.

Antibacterial properties of silver doped hydroxyapatite layers for medical applications

Over the last years it has become apparent that very few materials implanted in the body are truly biocompatible. Hydroxyapatite (HA) is one of the most frequently used materials in biomedicine. Its properties can be improved by dopation. To improve antibacterial properties of HA, silver can be used as a dopant. In collaboration with Faculty of Biomedical Engineering of Czech Technical University in Prague, we focused on investigation of minimum Ag concentration needed to reach a high antibacterial efficacy. Ag-HA films were formed by using laser. (Jelínek et al. Appl. Phys. A 101: 615-620, 2010; Jelínek et al. Laser Physics, 21:1265-1269, 2011; Jelínek et al. Mater. Sci. Eng., C 33:1242-1246, 2013; Kocourek et al. Laser Physics, 24, in press). We carried out *in vivo* measurement of antibacterial properties of HA, silver-doped HA and silver layers.

(4) Restriction-modification systems of Type I.

Type I restriction-modification enzymes are multifunctional heteromeric complexes with DNA-cleavage and ATP-dependent DNA translocation activities located on endonuclease/motor subunit HsdR. The HsdR subunit of Type I restriction-modification enzyme EcoR124I is an archetypical example of a fusion between nuclease and helicase domains into a single polypeptide. This linkage was found in many other DNA-processing enzymes.

Endonuclease and translocase activity of Type I restriction-modification enzymes

The recently described structure of the first intact motor subunit of the restriction enzyme EcoR124I allowed us to study the mechanism by which stalled translocation triggers DNA cleavage via a lysine residue on the endonuclease domain that contacts ATP bound between the two helicase domains. Using the molecular dynamics simulations we suggested that the Lys-ATP contact alternates with a contact with a nearby loop housing the conserved QxxxY motif that had been implicated in DNA cleavage. In a common project with a Institute of Nanobiology and Structural Biology ASCR at Nové Hrady, the interdomain communication in the endonuclease/motor subunit of Type I restriction-modification enzyme EcoR124I was proved (Sinha et al.; J. Mol. Mod. In press).

We participated in this study by the purification of the proteins, analysis of restriction function *in vivo* and mainly by an establishment of a unique system for analysis of translocation *in vitro*. We also contributed in characterisation of a number of mutant HsdR subunits published in Worldwide Protein Data Bank.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Cell Biology

2.1. Research on organized multicellular yeast populations

In the **Laboratory of Cell Biology (LCB)** the **research, mainly fundamental**, is focused on the biology of yeasts, mainly *Saccharomyces cerevisiae*. Thanks to its sequenced genome and well established methods of manipulation, *S. cerevisiae* is an excellent model for **studies of basic cellular processes that are highly conserved among different eukaryotes**.

The main focus of research in LCB concerns **yeast cells as constituents of highly organized multicellular communities, such as colonies and biofilms**. This research consists of two major research themes: studies of smooth colonies formed by laboratory strains of *S. cerevisiae* and studies of structured colonies, resembling biofilms that are formed by wild *S. cerevisiae* strains isolated from natural ecosystems. Colony structure and development differs significantly between the colony types, as does cell differentiation within colonies. This reflects the divergent needs of colony cell populations with very different lifestyles; i.e. favorable laboratory conditions and the more hostile natural environment. Both research themes are intensively studied in LCB, focusing particularly on the development of colony and cell morphology, physiology, metabolism, stress defense and adaptation, regulatory pathways and genome structures. The success of colonial yeast research benefits from long-term, close collaboration and the combined know-how of LCB and the "Yeast colony group" of Z. Palková (YCG) at the Faculty of Science, Charles University in Prague. Z. Palková initiated yeast colony research in the past with original finding that ammonia released by colonies affects subsequent colony development (published in Nature, 1997). Both labs are now **internationally recognized leaders in the field of yeast multicellularity**. This is a highly complex research area, covering a wide spectrum of different cellular processes and LCB members (except where otherwise stated) concentrate mainly on techniques, connected with the detection of protein production (including the identification of protein modifications), enzymatic activities, metabolites (e.g., amino acids), autophagy and other degradative pathways (proteasomes, vacuoles), stress defense mechanisms, specialized plasmid constructions, etc. The LCB team also provides vital know-how, such as an in-depth knowledge of biochemistry and microbial physiology. The YCG covers, for example, transcriptomics and proteomics analysis, analysis of signaling components (including small metabolites) and micromanipulation techniques, requiring specialized equipment. The two labs have jointly **developed and used unique techniques** of colony sample preparation, enabling the study of cellular processes *in situ* within the colony structure, using state-of-the-art two-photon confocal microscopy (2PE-CM) or using separated cell subpopulations. **These techniques allowed to identify unique features of specifically localized cell subpopulations and their spatio-temporal function within differentiated colonies.** The success of the teams' research in yeast colony biology and the benefits of intense collaboration between the labs is documented **in**

27 joint publications (total IF=141.126), 15 of them (total IF = 72.851) have been published within the evaluated period.

In the **research, focused on the development and differentiation of smooth colonies** formed by laboratory strains, we made **breakthrough discoveries regarding the unique metabolic and regulatory properties of the two major cell subpopulations,** present in differentiated colonies passing through alkali developmental phase, regulated by ammonia signaling. These two sub-populations are L cells, localized in the lower layers and U cells, localized in the upper layer of central parts of these colonies (Cap et al., ***Mol. Cell*** 46: 436, 2012; Vachova et al., ***Oxidative Med. Cell. Longev.***: ID 102485, 2013, Palková et al., ***FEMS Yeast Res***. 14: 96, 2014). We showed that L cells behave like stressed ROS-producing cells with active mitochondria, while U cells are able to reduce mitochondrial activity and oxidative stress, maintain high levels of intracellular glutamine, produce ammonia, undergo autophagy, re-activate the TOR pathway and maintain their vitality and the ability to proliferate even in 50 day-old colonies. Physiology and properties of U cells identified so far suggest that these **cells acquire unique metabolic and nutrient sensing properties which allow these cells to survive long periods of starvation**. Following up on similarities and differences in U/L differentiation in giant colonies and more quickly developing microcolonies, we showed that the formation of U cells and acquisition of their specific properties depend on active reprogramming of cellular metabolism and on ammonia signaling. L cell features, such as stress sensitivity are more dependent on colony age. Altogether our findings revealed **an unexpected similarity between colonial U cells and mammalian tumor cells and also a similarity between L cells and some tissues of mammalian organism affected by a tumor.** These findings (that have been greeted with great interest by researchers in tumor biology) pave the way toward using differentiated yeast colonies as a model system for the investigation of novel regulatory mechanisms involved in age-defense, longevity and possibly in tumor formation and development. As indicated above, the experimental work was split between the collaborating labs and model schemes were developed together during intense discussions, using complementary know how. For example, analyses of concentrations of amino acids and some other metabolites, the cell capability to respire and the activity of autophagy in cells located in particular cell subpopulations have been performed in LCB which also contributed to the monitoring of TORC1 activity in U cells. Resulting experimental data have been indispensable for designing a model of the flow of nutrients and regulatory compounds between U and L cells and proposing regulatory pathways, possibly orchestrating colony U/L cell differentiation.

Other important findings reached during the evaluated period that are related to the **investigation of smooth colonies** are listed below. These include identification of the important **role of the Jen1p carboxylic acid transporter** during the alkali phase of colony development (Paiva et al., ***Biochem J***., 454: 551, 2013). This finding fits with the above-mentioned proposed nutrient flow between U and L cells, which also includes the cycling of carboxylic acids. Furthermore, in this research we identified the presence of a novel transport activity of a currently unidentified lactic acid facilitator in colonial yeast cells. Members of LCB, together with YCG, performed most of the experimental work involved in these findings, both in Prague and in Portugal (linked with the stay of Dita Strachotova). The Portuguese partner helped with designing and performing lactate transport assays. We also revealed **interaction among ammonium exporters Ato1p, Ato2p and Ato3p** (important

players in colony development and differentiation), the interaction of which can regulate activity of these exporters (Strachotova et al., ***BBA-Biomembranes*** 1818: 2126, 2012). In this work we (at LCB and YCG) implemented an approach that allowed us, for the first time, to use combined FRET, FLIM and cell photobleaching to detect interactions between proteins in the membranes of cells as small as yeast cells. The group of J. Heyrovský Institute of Physical Chemistry of the ASCR helped with technical settings for FLIM and FRET measurements. With the aim of being able to follow **changes of intracellular pH in cell subpopulations during colony development,** we introduced a novel approach for the determination of intracellular pHi at the organelle level in living cells expressing pH-dependent ratiometric fluorescent proteins, using previously unreported wavelength shifts in pHluorin emission. This approach has already enabled us to identify the existence of a significant pHi gradient between the peripheral and internal cytoplasm of cells from colonies, occurring during the ammonia producing alkali developmental phase (Pineda Rodo et al., ***PLOS One 7***: e33229, 2012).

Breakthrough findings in research, focused on the development and differentiation of **structured biofilm colonies,** regards **uncovering of internal spatio-temporal architecture and differentiation** of biofilm colonies and identification of **multiple mechanisms** that provide differentiated cells of these colonies **complementary protection against the environment** (Vachova et al. ***J Cell Biol***. 194: 679, 2011). For example, layers of stationary-phase cells on the colony surface activate multidrug resistance (MDR) transporters, Pdr5p and Snq2p that are able to expel toxic compounds, while cells in the colony interior produce semi-permeable extracellular matrix (ECM) that very effectively protects matrix-embedded dividing cells. The whole three dimensional colonial structure is supported by thin fibers connecting the cells. Altogether, our findings suggest that a biofilm yeast colony is a finely tuned, complex multicellular organism in which specifically localized specialized cells jointly execute multiple protection strategies in colony cell population. Most of the experimental work was performed by LCB and YCG groups. Collaborators from the Institute of Physiology AS CR contributed settings for 2PE CM, provided in their Core facilities. The data in this paper together with findings presented in some other of our papers (Stovicek et al. ***Fungal Genet Biol***. 47: 1012, 2010 and Vopalenska et al. ***Environ Microbiol*** 12: 264, 2010) allowed us to identify the **production of extracellular matrix and adhesin Flo11p** to be the crucial **factors, essential for the formation of structured colonies**, i.e. for the lifestyle most likely to be adopted in the wild. We (LCB and YCG) also discovered that in the early stages of structured colony development yeast cells are able (contrary to the generally accepted assumption), to perform dimorphic transition and form pseudohyphae, independently of the adhesin Flo11p. Such pseudohyphae formation is induced by volatile ammonia produced by adjacent microcolonies, which results in oriented pseudohyphal growth toward an adjacent microcolony. Recent findings also revealed major changes that occur within cells during the **process of so-called "domestication"**, i.e. the process whereby some cells of a wild yeast strain reprogram and start to form smooth colonies. In LCB we succeeded in forcing **domesticated strains to switch back to a wild phenotype** and, together with YCG group, carried out extensive analyses of wild, domesticated and feral strains (Stovicek et al. ***BMC Genomics*** 15: 136, 2014).

The attractiveness of our research on yeast multicellularity to other groups worldwide can be demonstrated by **numerous invitations to write review papers discussing different aspects of yeast multicellularity** (Vachova and Palkova, ***Biochem Soc***

Trans. 39: 1471, 2011; Cap et. al., ***Oxidative Med. Cell. Longev.*** Volume 2012, ID 976753, 2012; Vachova et al., ***Oxidative Med. Cell. Longev.*** Volume 2012, ID 601836, 2012, Palkova et al., ***FEMS Yeast Res***. 14: 96, 2014). These reviews follow up previous invited reviews (e.g. Palkova and Vachova, ***FEMS Microbiol. Rev***., 30: 806, 2006; Vachova and Palkova, ***FEMS Yeast Res****.,* 7: 12, 2007). Our findings and implementation of new methodologies in yeast colony research also induced **new interactions with different research groups in Europe and USA** which will result, or have already resulted, in joint papers or new joint grant projects. For example in a joint paper (Cottier at al., ***PLOS Pathogen*** **8**: e1002485, 2012) published in collaboration with colleagues from the University of Kent, United Kingdom, LCB and YCG teams succeeded in visualizing, for the first time, the impact of CO_2 build-up on gene expression in entire fungal populations with an exceptional level of detail, achieved by the use of advanced microscopy. In 2014, in collaboration with G. Gilfillan from the Norwegian Sequencing Centre at Oslo University Hospital, LCB and YCG teams succeeded in obtaining grant support from the Czech-Norwegian Research Programme for using next generation sequencing technology in studies of differentiated yeast colonies (see also Research Plan).

2.2. Research on yeast liquid populations

In LCB, yeasts are also studied as individual cells in liquid cultures to investigate fundamental biological processes. We obtained new insights into the interplay among mitochondrial morphology, the respiratory activity of mitochondria and membrane potential during yeast chronological aging in batch cultures (Volejníková A. et al., ***FEMS Yeast Res***. 13: 7, 2013). This work was performed only in LCB.

Furthermore, we achieved novel findings (in collaboration of LCB and YCG with participation as mentioned above), supporting the hypothesis that SUN family proteins (Uth1p, Sun4p and Sim1p) are involved in yeast cell wall remodeling during the different phases of yeast culture development and under various environmental conditions. The finding that Uth1p is involved in cell sensitivity to boric acid, a commonly used antifungal compound in mycoses, opens up new possibilities for investigating the mechanisms of boric acid's action (Kuznetsov et al., ***PLoS One***. 8(9): e73882, 2013).

2.3. Research connected with other microorganisms

Both theoretically and practically important results were obtained in the exploration of the molecular properties of neutral cell lipids, namely triacylglycerols (TAGs) (in bacteria, algae, cyanobacteria, diatoms, and yeast), which are being increasingly studied as compounds with a variety of uses in nutrition, medicine, cosmetics, and as highly useful components of biofuels. We used a combination of state-of-the-art HPLC and MS techniques to identify and characterize for the first time the properties of a number of molecular species of TAGs. The research was conducted in close cooperation with the Laboratory of Fungal Genetics and Metabolism of the Institute of Microbiology AS CR, v.v.i. and parts of it were performed in cooperation with the Institute of Biotechnology, University of Chemistry and Technology, Prague. LCB (K. Sigler) participated by supervising its biological side (the study being otherwise mostly analytical), interpreting the data and writing up the manuscripts. The data were published in several papers (Řezanka T. et al., *Chem. Phys. Lipids* 163, 373 (2010), Schreiberová O. et al., *Lipids* 45, 743 (2010), Řezanka T. et al., *Phytochem*. 72*,* 1914 (2011), Řezanka T. et al., *Phytochem*. 72, 2342 (2011) *,* Řezanka T.et al.,

Phytochem. 78, 147-155 (2012), Řezanka T. et al., *Phytochem*. 80, 17-27 (2012), Kolouchová I. et al., *J. Separ. Sci.*36, 3310-3320 (2013), Řezanka T. et al., *J. Am. Oil Chem. Soc.* 90, 1327-1342 (2013), Řezanka T. et al., *Phytochem.* 104 (2014), Řezanka T., Sigler K. *Lipids* 49, 1251-1260 (2014)).

2.4. Applied research

Our knowledge of yeast properties and physiology also yielded **significant achievements in applied research** within the framework of the grant project "Use of immobilized yeast in biotechnology: the development of new applications for industrial processes" supported by the Technology Agency of the Czech Republic (collaborators of the project: Institute of Organic Chemistry and Biochemistry ASCR, v. v. i., YCG team, **LCB**, Institute of Physiology ASCR, v.v.i. and LentiKat´s a.s.). We contributed substantially to the optimization of Lenticats Biocatalyst for use with immobilized yeast (Functional sample "Optimized Lenticats Biocatalyst with immobilized yeast *Saccharomyces sp*., No. LentiKats-2013-01). We also participated in the design of a new genetically modified yeast strain, the cells of which turn red in the presence of copper ions, as the active component of a new biosensor (Patent application PV 2014-269). This strain has been applied using certified methodology (LENTIKVAS-2014-01) "Methodology of using the disposable kit for detection of the content of cupric (Cu^{2+}) ions in drinking waters and industrial wastewaters" by the company LentiKat´s a.s.

In collaboration of LCB with Research Institute of Brewing and Malting we developed a certified methodology (RIBM ISBN 978-80-86576-54-1) "Methodology for determining the effect of osmotic stress on the physiological state of brewer´s yeast and the course of main fermentation".

Scientific team of LCB

The LCB team includes junior members, such as master and doctoral students, and early to middle career scientists. The team also benefits from the experience of senior scientists (some of them working part-time), who are involved in the training of more junior members. In 2015 the team will be strengthened by the arrival of a new postdoctoral scientist. The team composition facilitates the efficient dissemination of knowledge from senior scientists, with extensive experience in yeast molecular and cell biology. In particular, group leader L. Vachova and junior scientist O. Hlavacek, have wide experience in specific techniques of yeast colony research, and are ideally placed to train junior postdocs and students with different level of experience. On other hand, new postdocs coming to the lab bring new expertise (gained either during their PhD studies in other research fields or during short-term stays in other specialized labs) useful for future progress in LCB research. For example postdoctoral scientist S. Resetarova has been recently trained in methods of protein immunoprecipitation and pull down techniques during her 3 month stay in the lab of J. Thevelein in Belgium (stay in frame of project IMPULS, Inovation in Microbiology - Postdoctoral training and laboratory center). New methodological knowledge that she obtained is very useful for the identification of regulatory complexes possibly participating in regulating the formation of specific cell subpopulations of yeast colonies. The balance of skills and personnel provides the LCB with excellent prospects for future research work.

Team composition in 2014
RNDr. Libuše Váchová, CSc. (head)
Doc. Ing. Alena Pichová. CSc. (senior scientist)
Ing. Otakar. Hlaváček, PhD. (junior scientist)
RNDr. Stanislava Rešetárová, PhD. (postdoc)
Alexandra Pokorná (technician)

Part-time staff

Ing. Karel Sigler, DrSc. (senior scientist)
Prof. RNDr. Zdena Palková, CSc. (senior scientist)
RNDr. Helena Kučerová (scientific assistant)
Ing. Markéta Begany (scientific assistant)

Students (all part-time staff)
Mgr. Karel Harant (doctoral)
Ing. Andrea Švenkrtová (doctoral)
Ing. Pavlína Pustowková (doctoral)
Bc. Lenka Belicová (master, later doctoral)
Bc. Veronika Očková (master, later full-time scientific assistant)
Bc. Lucie Čtvrtečková (master)

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Biology of Bacterial Pathogens

PRESENTATION OF THE MAIN DEVELOPED RESEARCH TOPICS

To the major achievements of our young and dynamic team **belongs** its **progressive transformation** from the Head + team structure into a pyramidal multi PI lab structure that consists of subteams headed by senior staff scientists. These interact very closely and work together **as a 'confederation of PIs', still steered and coordinated by the Head of the Laboratory. This structure gives the senior scientists (PIs) a high degree of freedom in chosing topics and conducting fairly independent research, while still allowing to maintain the necessary cohesion of the research efforts and operation of an integrated team**. It means that besides the Head, who was PI on 6 grants over the evaluated period (2010-2014), other **5 senior researchers of the lab successfully applied for grants and became PIs on their own projects** funded by the Czech Science Foundation (4 – Drs. Bumba, Mašín, Osička and Večerek) and of Ministry of Agriculture (Dr. Staněk). **Each of the PIs is now supervising a small group of master and doctoral students**, managing his subteam and focusing on selected topics. **This is considered as a particular strength.**

Over the evaluation period, our team produced 20 own papers that we consider as our core work. We have participated on additional 23 papers of other teams. Altogether this generates a **large body of novel and highly relevant scientific knowledge** that was published in **43 peer-reviewed publications.** Their full list is attached at the end of this report and includes a number of papers in highly reputed journals like **PLoS Pathogens, Molecular Microbiology, Infection and Immunity, Journal of Immunology, Mucosal Immunology, Nucleic Acids Research, RNA biology and Journal of Biological Chemistry.**

The central focus of research developed in our lab is on the molecular basis of action of bacterial virulence factors. **In particular, we kept focusing on deciphering of molecular mechanisms of receptor and target cell binding, on membrane penetration and on immunosubversive signaling in phagocytes of the adenylate cyclase toxin (ACT) from *Bordetella pertussis*.** This represents the central historical 'core focus' of our research activities**, for which we are internationally recognized as international leaders in the respective field.** This translates into frequent invitations to give lectures at international meetings and renowned foreign academic institutions (see attached list for 2010-2014)**.**

Besides the core research activity on ACT, we developed a certain number of new projects aiming at transforming the acquired mechanistic insight into novel tools for antigen delivery into dendritic cells for presentation to T cells and for development of novel T cell vaccines. Besides that we developed an array of additional research activities, directed by senior scientists of our team. This enabled to transform our laboratory into a highly over-solicited training site for doctoral students that can obtain a very broad and interdisciplinary scientific culture while working inside of a rather diverse and broad research team. We hold weekly labmeetings of the entire team, where work progress is presented by individual team members. This enables the students and researchers to learn from each other and to maintain a high level of awareness on who is doing what and by which methods, thus cross-fertilizing

research collaborations inside of our fairly large team. The spectrum of the used methods then ranges from reverse bacterial genetics, transcriptomics and RNA work on gene regulation, to structural biology, biochemistry, signaling pathway analysis, molecular and cellular biology of pathogens, up to cellular immunology and vaccine-related research. This environment offers a unique opportunity to our trainees to get a quite broad and complete scientific culture and to acquire a number of technical skills useful for all kinds of biomedical research careers.

THE RESEARCH ACTIVITIES OF THE TEAM OVER THE EVALUATED PERIOD (2010/2014) CAN BE ROUGHLY BROKEN DOWN INTO THE FOLLOWING 5 SUBTOPICS (ST):

ST1 Structure, function and molecular mechanism of action of adenylate cyclase toxin (ACT) from the whooping cough agent *Bordetella pertussis,* which comprises:

1) *Structural basis and molecular mechanisms of CR3 receptor recognition by ACT (Dr. Osička)*
2) *Membrane interactions and pore-forming activity of ACT (Dr. Mašín)*
3) *cAMP signaling of ACT in phagocytes molecular mechanisms of subversion of signaling pathways of phagocyte towards ablation of their bactericidal capacities (Dr. Šebo)*
4) *Structure of the RTX domain and molecular mechanism of secretion of ACT through the T1SS assembly (Dr. Bumba)*

ST2 Other virulence mechanisms of *B. pertussis* and role of sRNA in regulation of the T3SS expression and function, which comprises:

1) *sRNA regulation of virulence gene expression in B. pertussis (PI = Dr. Večerek)*
2) *Expression and biological role of the T3SS system of B. pertussis (PI = Dr. Večerek)*
3) *Structure, function and immunosuppressive activity of B. pertussis FHA (Dr. Šebo)*

ST3 Mechanisms of action of other virulence factors of other bacterial pathogens

1) *RTX proteins FrpC and ApxIVA* of *N. meningitidis* and *A. pleuropneumoniae and structure, role in virulence and* mechanisms of their self-processing action *(Dr. Bumba)*

ST4 Antigen delivery tools and technologies based on ACT and streptavidin fusions (dr. Staněk)

ST5 Contract research on development of novel pertussis vaccines (confidential)

SUMMARY OF MOST IMPORTANT RESULTS AND ACHIEVEMENTS IN 5 YEARS.

Given the substantial volume of scientific production of our team, only the key results mostly achieved on ST1 and focusing on adenylate cyclase toxin structure and mechanism of action were selected for being discussed in more detail below. These represent the achievements that

we ourselves value most for their novelty and for the extent to which these results advanced our knowledge and understanding of the studied mechanisms. For the rest of our scientific production the reviewer is kindly referred to the abstracts of our other publications.

ST1 Structure, function and molecular mechanism of action of adenylate cyclase toxin (ACT) from the whooping cough agent *Bordetella pertussis*:

In 1999 we have proposed a model of ACT action, which predicted that ACT adopts 2 mutually independent conformations. One would yield an AC domain-translocating and membrane-inserted precursor that would deliver the enzymatic adenylate cyclase domain into phagocyte cytosol to provoke cAMP-mediated signaling. The other conformation would yield a pore precursor that would oligomerize into cation-selective ACT pores enabling potassium ion leakage form phagocytes and accounting for the hemolytic activity of ACT. After almost 15 years of intense effot we were able to obtain a combination of two residue substitutions (E570K+K860R) that yielded an ACT protein almost devoid of any pore-forming activity, while it still translocated the AC domain fully across the plasma membrane of target cells to catalyze ATP to cAMP conversion in cells normally (**Osickova *et al.* 2010, *Mol. Microbiol.* *75,* 1550-1562**). With the help of French colleagues (Drs. Fayolle and Leclerc), we could show that the toxoid derived from such mutants still delivers foreign epitopes into cytosol of dendritic cells, while not permeabilizing their plasma membrane (patch-clamp measurements in collaboration with Dr. Krůšek). **These results established that ACT monomers have the capacity to translocate the AC domain directly across host cell membrane lipid bilayer without transit of the AC across the oligomeric ACT pore and that the AC domain delivery and oligomeric pore formation activities are two fully parallel and independent activities associated with two different conformational forms of a single protein. These results set a new paradigm for the entire field of research on protein toxin translocation across target cell membranes.**

XXX

As a seminal discovery (**Bumba *et al.* 2010, PLOS Pathogens 6 (5),| e1000901**), we have established that translocation of the AC enzyme domain of ACT across the plasma membrane of phagocytes occurs in two steps. We showed that upon binding the CR3 receptor (CD11b/CD18), the toxin inserts into cell membrane and forms a conduit through which extracellular calcium ions penetrate into submembrane space of the cells. As a result, the protease calpain is activated and cleaves talin that tethers the CR3 receptor to actin cytoskeleton. This liberates the toxin-receptor complex for lateral relocation into the cholesterol-rich lipid microdomains (rafts) of the membrane. We could then show that in a second step the AC domain translocation is accomplished from the lipid rafts across the cholesterol-rich membrane. **This mechanistic insight allowed us to propose a refined model of ACT action on phagocyte membrane that sets a new paradigm for the entire field.** Physiologically, the translocation of the AC domain across lipid rafts would yield cAMP generation directly inside the cellular 'signalosome' structures beneath lipid rafts, where the cAMP-activated PKA is located, thereby maximizing output of toxin action in subverting host cell signaling.

In a subsequent paper (**Holubova *et al.* 2012, Infect. Immun. 80:1181-92**) we could show, by using deletion mutagenesis and insertion of foreign T cell polyepitope sequences, that the AC domain segment of N-terminal 374 residues is only a passive passenger that is translocated across the target cell membrane by the RTX hemolysin moiety. We could show that synthetic polyepitope constructs, can be used to replace the AC domain polypeptide and will still be delivered into CD11b-expressing dendritic cells for presentation on MHC class I

molecules and for stimulation of antigen-specific human cytotoxic $CD8^+$ T lymphocytes. **These results are highly relevant for construction of next generation of vaccines for cancer immunotherapy that employ the T cell antigen delivering capacity of detoxified ACT.** Indeed, we have a long history of developing the technology based on using ACT as antigen delivery tool and have previously contributed to development of toxoids that are currently undergoing Phase II clinical trials, being tested as potential therapeutic vaccines for HPV-induced cervical cancer in woman (www.genticel.com).

Finally, using a particular set of ACT toxoid mutants we were able to show that it is the calcium influx-provoking insertion of the ACT precursor into target cell membrane that decelerates the removal of toxin-CR3 complexes form cellular membrane by clathrin-mediated endocytosis. This extends the half-life of ACT molecules present in plasma membrane of the cells and promotes their oligomerization into pores that enable efflux of potassium ions form cells, which further decelerated endocytosis of toxin into cells and yields exacerbation of cell permeabilization by toxin pores by a positive feedback loop mechanisms contributing to cytotoxic action of ACT (**Fiser *et al.*2012, PLoS Pathog 8(4): e1002580**). **This work again set a new paradigm for the entire field of pore-forming toxin research.**

Mapping of the pore-forming domain of ACT then led us to the discovery of a threshold of pore size that needs to be reached in order to observe the 'Purinergic Amplification of Osmotic Cell Lysis by the Pore-Forming RTX Toxins'. We showed that toxins forming pores of differing size do or do not employ this amplification mechanism (**Masin *et al. Infect Immun. 81:4571-4582***).

Further research on structure function relationships underlying ACT secretion and receptor binding led us to two important discoveries that will be published during this year. The subteam of Dr. Osička has constructed a series of molecular chimeras of the CR3 and CR4 heterodimeric complement receptors and showed that ACT binds its CD11b subunit by electrostatic interactions outside of the major ligand binding site of CR3 (CD11b/CD18). This defined a completely novel binding site near the hinge region of the bended $\alpha_M\beta_2$ integrin molecule. We could further show that subsequent translocation of the AC enzyme into vicinity of cytosolic tail of the integrin molecules and the production of cAMP does yield dephosphorylation and inactivation of the Syk kinase by an as yet unidentified tyrosine phosphatase. This then yields a near instant ablation of oxidative burst and complement-mediated opsonophagocytic capacities of monocytes, thus subverting host innate defenses. The manuscript summarizing there breakthrough results is currently **being revised for submission into *Proc. Natl. Acad. Sci. USA*.**

Another set of results going far beyond the state of the art was achieved by the subteam of Dr. Bumba. The large body of generated cutting-edge data of Dr. Bumba explains the molecular mechanism of excretion of the whole RTX leukotoxin protein family from Gram-negative bacteria, where the proteins transit directly from bacterial cytosol into the extracellular space across both the inner and outer bacterial membranes in a single step, using the T1SS nanomachines and the unprocessed C-terminally located secretion signals. The manuscript summarizing these data is written up and **will soon be submitted into the journal *Cell* for evaluation**. It is probably the biologically most generally relevant set of data we ever generated. In brief, we have solved the 3D structure a C-terminal segment of ACT both by X-ray crystallography, as well as by NMR spectroscopy. We showed that the segment contains a folding nucleus that promotes calcium-binding folding of RTX repeat blocks into β-barrel structures once exiting form the outer mouth of the T1SS assembly on bacterial surface. Finally we show that these structures form Brownian molecular ratchets that prevent backsliding of the translocating RTX polypeptide in the T1SS channel tunnel conduit

assembly that spans across the bacterial cell envelope. **We could thus propose a new 'push-pull' mechanism of T1SS-medoiated RTX protein family excretion.**

Last not least, in the past years we achieved a real breakthrough in analysis of the mechanisms by which ACT-generated cAMP signaling subverts bactericidal capacities of host phagocytes. We showed that *Bordetella* adenylate cyclase toxin differentially modulates Toll-like receptor-stimulated activation, migration and T cell stimulatory capacity of dendritic cells (**Adkins *et al.* PLoS One 9(8):e104064**). We showed that ACT subverts TLR-induced maturation of DCs and enhances their chemotactic migratory capacities. In the next years we shall thus test the hypothesis in a baboon model of *Bordetella* infection, that ACT action on intraepithelial DCs of infected airway mucosa yields migration of tolerogenic DCs into draining lymph nodes. We hypothesize that this leads to suppression of pathogen-specific adaptive T cell immune responses against the whooping cough agent and thus promotes extended bacterial colonization.

In more mechanistic studies on subversion of host cell signaling by ACT, **we have recently discovered a new signaling axis through which toxin-generated cAMP and activation of protein kinase A (PKA) results by an as yet unknown mechanism in activation of the tyrosine phospahatase SHP-1 that regulates a whole number of key immune immunity mechanism in leukocytes.** We show that SHP-1 activation and AP-1 transcription factor dephosphorylation results in **inhibition of inducible nitric oxide synthase expression in phagocytes, which extends survival of intracellular B. pertussis bacteria in macrophage cells.** The work will soon be published in Journal of Immunology (C**erny *et al.* 2015, The Journal of Immunology, 2015, 194: in press**).

Another important contribution of our team to the research in the field is currently under revision for the journal *Cellular Microbiology* (**Ahmad *et al*. 2015, *Cell. Microbiol*.**). We have discovered, that **cAMP signaling and PKA-mediated activation of the SHP-1** phosphatase, **together with inhibition of the ERK1/2 and JNK** kinases yields stabilization of the proapoptotic protein BimEL. This activates Bax and **triggers mitochondria permeabilization-dependent apoptosis** of toxin treated macrophages, which **subverts innate immune defenses on *B. pertussis*- infected host airway epithelia.**

ST2 Other virulence mechanisms of *B. pertussis* and role of sRNA in regulation of the T3SS expression and function:

A new research activity in our lab was developed by Dr. Vecerek, who joined the lab in 2010 and started research on the role of sRNA in regulation of expression of virulence factor genes of *Bordetella pertussis*. He has built up a very dynamic and successful subteam inside the lab that focuses on transcriptomics and RNA research. His subteam was able to show that the RNA chaperone Hfq plays a major role in sRNA-mediated postranscriptional regulation of *B. pertussis* virulence (**Bibova *et al.*, 2013, *Infect Immun.* 81:4081-4090**). More recently, this subteam could show the role of sRNA in regulation of the T3SS component expression of *B. pertussis* (**Bibova *et al.*, 2015, *RNA Biol.* 2015, Feb;12(2):175-85**).

After having been incubated in our lab for 5 years, the subteam of Dr. Vecerek will now be spun-off into the newly opening research center BIOCEV, where it will form a new independent research group by the end of 2015.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Bioinformatics

The laboratory combines **bioinformatics and computational biology** approaches to the analysis **of large scale gene expression and sequence data**. For this purpose, number of computational models and analytical tools were developed and applied to the data that were either obtained from public resources, trough collaborations with other labs within and outside the institute, or generated within the wet laboratory part of the lab.

In the respective period, the laboratory went in two principle, mutually complementing, directions. The first focussed on the **computational modelling of cell regulatory systems** with a special focus on transcriptional and posttranscriptional regulation. Second direction focussed on the **analysis of RNA sequences** with respect do their role in translation and posttranscriptional control. The ultimate goal is the development of models and analytical tools for inference and description of gene expression networks on transcriptional, posttranscriptional and translational levels and uses them in systems level analysis of developmental and/or evolutionary processes.

The first part was based on the **neural network model of gene expression which was developed in our lab** and extended to inference of complex genetic networks. The early paper of 2010 [1] dealing with medium sized model genetic network (yeast cyclins network) analysed quantitative aspects of genetic networks topology and revealed association between accuracy with which the gene expression can be measured and the different topologies of the network. The idea of influence of gene expression levels on network topology was extended in the later theoretical paper [2] in which a quantitative model of a genetic network was subjected to virtual gene deletion and the propagation of this perturbance was followed along the network. The results showed that the quantitative part, i.e. the amount of expressed mRNA can influence propagation of the deletion through the network, and showed that causally connected nodes (genes) of the network may in, principle, not be influenced by the deletion of the upper genes, or may go in different directions depending on the quantitative parameters of the nodes of the network.

The direction of the theoretical modelling of gene expression control was extended in a 2012 paper [3] on **stochastic simulation of gene expression**. There we presented an algorithm that simulates stochastic behaviour of individual molecules participating in transcriptional control. Using binding data for transcriptional regulators, we simulated all possible combinations of the genes of a model network of yeast cyclins and showed interactions that are in accordance/discordance with the principles of molecular interactions leading to gene transcription. The work confirmed, on a molecular level, that simple binding data are not sufficient to describe functional interactions between regulator and regulated gene, and, in order to find interactions active during an observed physiological process, they have to be complemented with kinetic measurements and modeling.

A model, based on the kinetics of gene expression, binding of miRNA, and a decay of the complex, was developed and tested on temporal microarray data of HepG2 cells transfected with miRNA-124a [4]. Using the model, our analysis revealed a **novel mechanism of mRNA accumulation control by miRNA**, predicting that specific mRNAs are controlled in a digital, switch-like manner. Specifically, the contribution of miRNAs to mRNA degradation is switched from maximum to zero in a very short period of time. Such behaviour suggests a model of control in which mRNA is at a certain moment protected from binding of miRNA and further accumulates with a basal rate. Genes associated with this process were identified and parameters of the model for all miRNA-124a affected mRNAs were computed.

The kinetic modelling of gene expression was further adopted for a systems level analysis of a process with defined origin – **germination**. As a model organism we used *S. coelicolor* a bacteria whose germinating period can be used, for its complexity, as a template for other organisms and which was largely ignored due to the fact that the antibiotic biosynthesis, which has attracted most of the attention, occurs later in the growth. For this reason we have conducted large scale experiment covering first 5.5 hours of growth in 13 time points, when we collected mRNA and concomitantly protein samples that were quantified by microarrays for mRNA and 2DE, radiolabeling and MS for proteins. This arrangement allowed us not only to track kinetics of accumulation of gene expression, rate of protein expression, and mRNA accumulation, but also compare gene and protein expression on a global scale. Resulting paper [5] analysing time series of gene expressions using correlation, principal components analysis and an analysis of coding genes utilization, revealed that the global trends in the gene/protein expression reflected by the first principal components are both in the protein and the mRNA domains surprisingly well correlated. In contrast with the correlation analysis, that showed only limited correlation, the PCA, which can discover principal trends underlying the observed process, was able to identify processes and pathways that may be considered as core, controlling whole process of germination.

The germination expression data were further analysed individually on protein [6] and mRNA [7] levels. The protein level analysis showed that the full competence of spores to effectively undergo active metabolism is derived from the sporulation step, which facilitates the rapid initiation of global protein expression during the first 10 min of cultivation. Within the first hour, the majority of proteins were synthesized. From this stage, the full capability of regulatory mechanisms to respond to environmental cues is presumed. The obtained results might also provide a data source for further investigations of the process of germination. The mRNA analysis focussed on, first – the influence of the sigma factors on the regulation of expression of genes during germination [7]. For this purpose we adopted our previously developed computational model of gene expression (see above) and identified genes and gene clusters that might be controlled by the identified sigma factors. Using genome sequence annotations, functional groups of genes that were predominantly controlled by specific sigma factors were identified. Using external binding data, complementing the modelling approach, specific genes involved in the control of the studied process were identified and their function suggested. The analysis led to construction of large scale network of sigma factor controlled genes and gene clusters.

Differential analysis of gene expression levels in individual time points during germination [8] revealed genes and pathways activated during each individual periods of germination. The analysis also evidenced that majority of mRNAs are

already stored in the spores and after rehydration they serve to initiate the growth. After that, they are degraded and resynthesized later at the time when they are necessary for growth. **Specific role of sigma factors** and their activation during germination was identified. From the analysis of sigma factor response, we conclude that conditions favouring germination evoke stress-like cell responses.

Bioinformatics analysis of RNA sequences focused on: 1) identification of **small regulatory RNAs (sRNAs) and prediction of function** of RNAs, 2) prediction of **mechanisms of function of RNA in regulation of eukaryotic gene translation**.

Ad. 1)

We computationally identified new homologs of small regulatory RNAs (sRNAs) in bacteria using suboptimal model of RNA secondary structure [9]. Biocomputational identification of homologs of known sRNAs in other species often fails due to weakly evolutionarily conserved sequences, structures, synteny and genome localization, except in the case of evolutionarily closely related species. Experimental discovery of sRNAs has almost always been either occasional or in a form of a secondary result. We reasoned that the structure is the only evolutionarily preserved property of homologous sRNAs as they do the same function in different organisms and showed that **suboptimal structure model is capable of capturing RNA homology even in divergent bacterial species**, in which similarity of other sRNA properties, including the MFE, i.e. optimal secondary structure, is extremely week or none. A computational procedure for the identification of homologous sRNAs using suboptimal structures was developed. It was applied to search for sRNA homologs in strongly divergent bacterial species and **identified previously unknown homologous sRNAs**. One of the predicted homologs showed itself as one of fundamental bacterial sRNAs, when its function was experimentally characterized in M. smegmatis by a collaborating lab [10].

Ad. 2)

Here we carried out two projects. The first one focused on **function of secondary structure of 5' UTR mRNA in eukaryotic translational contro**l in collaboration with Lab. of regulation of gene translation, Inst. of Microbiol., ASCR [11]. Using computational modeling of RNA structure supported by experimental data we suggested that, in order to act, the mRNA 5' UTR must progressively fold into specific local secondary structures, while the 40S ribosome scans through it. These local structures work in cooperation with uORFs, thus add another level into the eukaryotic translational control. We computationally predicted a mechanism of its function that was experimentally verified in the collaborating lab.

The other project aimed at computational search for distribution of **mRNA-rRNA sequence complementarity in both mRNA and rRNA sequences** [12]. We took advantage of robustly growing large-scale data sets of mRNA sequences for numerous organisms, solved ribosomal structures and used extended computational power to explore the mRNA–rRNA complementarity that is statistically significant across the species and which is evolutionarily conserved. Our predictions revealed a pattern of sequence complementarity in eukaryotic 18S rRNA sequences that forms an evolutionarily conserved, solvent accessible 3D pattern on the 40S subunit. Very specific properties of the pattern implied that 5’ UTRs of eukaryotic mRNAs, which have already emerged from the mRNA-binding channel, may contact several complementary spots on 18S rRNA situated at the solvent-exposed segments of 40S

ribosome near the mRNA exit pore and on the left ribosomal foot. This contact, potentially **establishing interaction between 40S and translated mRNA, may stabilize the mRNA on 40S and thus participate on regulation of the eukaryotic gene translation**.

Very recently, we found a pattern of mRNA-rRNA complementarity between 18S rRNAs and 3' UTRs (paper under review). This pattern was found in Metazoa, not in Protozoa. Its predicted function is to contribute to **stabilization of post-translational complexes on mRNAs, which is necessary for ribosomal recycling, and was positively experimentally tested** in the collaborating lab.

Additional information

The laboratory is involved in the large European infrastructures ELIXIR (ELIXIR_CZ) and ISBE (C4Sys), part of ESFRI and ERIC, in the framework of which, specific bioinformatics tools have been developed – **rPredictor** (http://rpredictor.ms.mff.cuni.cz/) a web based tool for modelling of secondary rRNA structures. This tool has been developed in collaboration with informatics department of MFF UK, Prague, where we provided the analytical algorithms and the collaborators developed the web environment. **SWICZ** (proteome.biomed.cas.cz), a web accessible proteomic database storing and disseminating data on protein expression time series and results of their analyses, for the streptomyces, caulobacter and meningococus geni. The data are linked with other resources as NCBI, KEGG, etc. The database was originally developed in collaboration with the Biozentrum of University of Basel, Switzerland, since 2005 it is fully maintained by our lab. **GenExp** (http://proteom.biomed.cas.cz/genexp/), a downloadable gene network simulation environment running under MATLAB.

Numbers of other modelling tools are part of published papers and are available upon request from the routines repository of the lab.

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13. Pánek J, Krásný, L., Bobek, J., Ježková, E., Korelusová, J., Vohradský, J.: **The suboptimal structures find the optimal RNAs: homology search for bacterial non-coding RNAs using suboptimal RNA structures.** *Nucleic Acids Res* 2010.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Cell Signalling

A) LABORATORY OF CELL SIGNALLING

The **Laboratory of Cell Signalling**, established in 2002 and headed by **Pavel Branny**, has over the past 13 years established a strong **team specialized in molecular bacteriology**. The Laboratory introduced a **new field of research** at the Institute of Microbiology focused on **signal transduction mechanisms**. In addition to our own research the Laboratory also collaborated both technically and intellectually with other groups. The findings emerging from these collaborations were either published or recently submitted for publication.

Laboratory of Cell Signalling is engaged purely in the **basic research studies** of signal transduction mechanisms that controls many vital cellular functions in **both Eukaryotes and Prokaryotes**. While the research performed in the laboratory is mainly fundamental of nature, it is to be expected that any knowledge on the signaling mechanisms will be highly relevant for the potential medical applications. Insights from this research might provide new ways to target a key control points and, subsequently, help in the design of innovative therapeutic compounds.

Student education and training is another important function of the laboratory. So far, **11 PhDs in molecular bacteriology** has been trained and **18 master thesis** have been elaborated in the laboratory. This signficantly contributes to education of highly skilled workers for both basic and applied research. Indeed, all mentored students pursue their career either at academic research institution or at biotech industry. In addition, from 2006 P. Branny teaches advanced semestral course „Bacterial genetics in experiments“ at Charles University (20% contribution).

The research in the Laboratory of Cell Signaling headed by **Pavel Branny** focuses on the molecular mechanisms that bacteria use for intra- and intercellular communication. Our goal is to understand how bacteria detect multiple environmental cues, and how the integration and processing of this information provided by eukaryotic-type Ser/Thr protein kinases results in the precise regulation of gene expression. Through reversible protein phosphorylation, protein kinases and phosphatases provide the fundamental machinery for environmental sensing and physiological signaling.

The human pathogen Streptococcus pneumoniae is an excellent model to study eukaryotic-like protein kinases in bacteria since its genome only encodes for a single Ser/Thr protein kinase StkP. Our prior studies demonstrated that StkP regulates cell division, virulence, competence, stress resistance and gene expression. Five substrates of StkP were identifed to date including cell division protein DivIVA. The central hypothesis of our research is that StkP senses concentration of free peptidoglycan subunits and in response modulates activity of group of substrates to coordinate cell division and cell wall synthesis in S. pneumoniae. The main objective of our work is to gain insight into the activation, localization and dynamics of StkP in vivo and to investigate the significance of phosphorylation of StkP substrates.

The proteomics group, headed by **Jaroslav Weiser** was completing their previous projects concerning studies of morphological and biochemical differentiation in Streptomycetes and biofilm structures formation in Mycobacteria. The group is currently engaged in main projects in the lab concerning molecular signalling in Streptoccoci.

The main focus of **eukaryotic research team** headed by **Tomáš Vomastek** has been on the architecture and regulation of the ERK cascade; in particular how is the ERK signaling, through the phosphorylation of functionally diverse substrates, implemented into specific biological response. After long-term postdoctoral stay in USA, T. Vomastek joined temporarily the Laboratory of Cell Signalling in order to get access to necessary laboratory infrastructure and to ease the process of reintegration and the transition phase to full independence. The establishment of the independent research laboratory is expected to formalize during the year 2016.

B) MOST IMPORTANT RESULTS ACHIEVED IN THE PERIOD OF EVALUATION

In prokaryotic cell signalling group supervised by P. Branny we achieved these important results:

Using immunodetection with specific anti-phospho-threonine antibody **we detected StkP substrates *in vivo***: **cell division protein DivIVA**, **hypothetical membrane protein Spr0334** and Mn dependent inorganic pyrophosphatase PpaC. DivIVA and Spr0334 were proved to be targets of StkP also *in vitro*. Pneumococcal DivIVA is **involved in cell shape determination**, **septum assembly, and completion,** as well as chromosome segregation through an unknown mechanism. We showed that **StkP activation and substrate recognition** depend on the presence of a **peptidoglycan-binding domain** comprising four extracellular PASTA domain repeats. Our finding that DivIVA is a substrate of StkP determined our focus on cell division in pneumococcus. This work (Nováková et al, 2010) was entirely done at Institute of Microbiology.

We sought to **define T-cell epitopes in the Ser/Thr kinase of S. pneumoniae (StkP)** and to investigate interferon g (IFN-g) production resulting from such T-cell activation in healthy donors. We found that the **majority of the T-cell responses** were directed **against** the extracellular, penicillin-binding protein and serine/threonine kinase-associated domains (**PASTA**) (Aslam et al., 2010). This work was done in the collaboration with Institute of Molecular Medicine, University of Oxford, United Kingdom. Our laboratory prepared all the expression constructs including particular epitopes.

We performed a spectroscopical characterization of StkP structure and stability. Biophysical experiments show that **C-StkP binds** to **synthetic samples of the cell wall peptidoglycan** and to **β-lactam antibiotics**, which mimic the terminal portions of the peptidoglycan stem peptide. This is the **first experimental report** on **the recognition of a minimal peptidoglycan unit** by a PASTA-containing kinase, suggesting that **non-crosslinked peptidoglycan may act as a signal for StkP function** (Maestro et al., 2011). This paper is the result of collaboration of three laboratories. Our laboratory expressed and purified extracellular part of StkP and performed binding assays. Researchers from University of Notre Dame (USA) synthetised synthetic peptidoglycan subunit. Collaborators from University of Miguel Hernandez (Spain) performed measurements. All three group leaders contributed equally to writing the paper.

We analysed the **transcriptome of the double Ser/Thr phosphatase-kinase pppA/ppkA mutant** of **Pseudomonas aeruginosa**, another model organism in our laboratory, under **conditions of oxidative stress** and showed an **impaired response** to the stress, manifested by

a **weaker induction of stress adaptation genes** as well as the genes of the **SOS regulon**. Our results suggest that in addition to its crucial role in controlling the activity of P. aeruginosa type-VI secretion system H1 at the post-translational level, the PppA-PpkA pair also affects the transcription of stress-responsive genes (Goldová et al., 2011). This work was entirely done at Institute of Microbiology.

In our further work we determined successfully the **role of the sensor domain of StkP** in signal transduction in relationship with other cell division proteins, and characterized activation signals in vivo. We performed in vivo protein-localization studies in live pneumococcal cells and investigated molecular determinants for localization of StkP and **dynamic localization of StkP** during pneumococcal life cycle and in response to extracellular stimuli. The data showed that **StkP** plays an important role in regulating cell-wall synthesis and **controls correct septum progression and closure.** In the absence of StkP, this control is lost: Cells often are elongated as a consequence of excessive peripheral cell-wall extension or a block in cell division. Overall, our results indicate that **StkP signals information about the cell-wall status** to key cell-division proteins and in this way acts as a **regulator of cell division**. These findings resulted in manuscript that was published in PNAS (Beilharz et al., 2012). This paper is the result of collaboration of three laboratories. Our laboratory invented the **key hypothesis** of this work and prepared most of the mutants. Both leading authors contributed equally to this work. Researchers from University of Groningen (The Netherlands) provided a key metodology. Collaborators from University of Cagliari (Italy) performed immunofluorescent microscopy. All three group leaders contributed equally to writing the paper.

Year later in invited **review** (Massidda et al., 2013) we discussed the recent **advances in understanding *S. pneumoniae* growth and division**, in comparison with the best studied rod-shaped models, *Escherichia coli* and *Bacillus subtilis*. Importantly, *S. pneumoniae* and close relatives with similar morphology differ in several aspects from the model rods. The data support a model in which a single large machinery, containing both the peripheral and septal peptidoglycan synthesis complexes, assembles at midcell and governs growth and division. We contributed equally to this review with collaborators from University of Cagliari (Italy) and Newcastle University (United Kingdom).

In the same year we published the work describing the **function of WD-40 proteins** in S. coelicolor (Ulrych et al., 2013). The whole-genome **transcriptome analysis** revealed that deletion of wdpB affects the expression of genes involved in **aerial hyphae differentiation**, **sporulation and secondary metabolites production**. Deletion of wdpC cause downregulation of several gene clusters encoding secondary metabolites. The results obtained suggest that both genes studied have a **pleiotropic effect on physiological and morphological differentiation**. This work was entirely done at Institute of Microbiology. Majority of the experimental work was done in our laboratory.

Besides **DivIVA**, we identified **new cell division protein**, named **LocZ** (**Spr0334**), which is involved in proper septum placement in *S. pneumoniae*. LocZ is a **substrate of protein kinase StkP** and is conserved only among streptococci, lactococci, and enterococci, which lack homologues of the Min and nucleoid occlusion effectors. We showed that *locZ* is not essential but that its deletion results in cell division defects and shape deformation, causing cells to divide asymmetrically and generate unequally sized, occasionally anucleated, daughter cells. **LocZ** has a **unique localization** profile. It arrives early at midcell, before FtsZ and FtsA, and leaves the septum early, apparently moving along with the equatorial rings that mark the future division sites. Consistently, cells lacking LocZ also show misplacement of the Z-ring, suggesting that it could act as a positive regulator to determine septum placement. We showed that LocZ is a new cell division protein important for **proper septum placement** and

likely functions as a **marker of the cell division site (**Holečková et al., 2014). Consistently, **LocZ supports proper Z-ring positioning at midcell**. Together, all these data indicate that ovoid bacteria adapted a **unique mechanism** to find their middle, reflecting their specific shape and symmetry. Further, they provide the evidence that the control mechanism in the StkP pathway is distributed between several StkP substrates, rather than being elicited by a single effector substrate. These substrates regulate distinct cellular functions such as biosynthesis of the cell wall precursors and cell division. Except for the electron microscopy (Group of electron microscopy of the Institute of Microbiology), all the experimental work was done in our laboratory.

The research of the **former proteomic group** supervised by **J. Weiser** resulted in these scientific achievements:

The occurrence of **phosphorylated proteins in ribosomes** of Streptomyces coelicolor was investigated. The ribosomal proteins were found to be phosphorylated mainly on the **Ser/Thr residues**. We also found that phosphorylation of the ribosomal proteins **influences ribosomal subunits association**. In addition our data demonstrate that phosphorylation of ribosomal proteins affects critical steps of protein synthesis (Mikulík et al., 2011). The work was done at Institute of Microbiology.

We provided the **first evidence** for **deacetylation activity of CobB1** in Streptomyces coelicolor. Using specific antibodies against acetylated lys, CobB1, and acsA, we found relationship between level of CobB1 and acetylation of acsA, indicating that **CobB1 is involved in regulating the acetylation level of acsA and consequently its activity** (Mikulík et al., 2012). Most of the experimental work was done at Institute of Microbiology.

For the first time we **demonstrated the effects** of two **different inert surfaces**, glass and zirconia/silica, **on the growth and antibiotic production** in Streptomyces granaticolor. The finding, that the quality of the surface influences quantitatively the production of secondary metabolites in streptomycetes, could be used in antibiotic screening programmes (Petráčková et al., 2013). The work was performed in collaboration with the Group of electron microscopy of the Institute of Microbiology.

Weiser's group studied the early stages of pellicle formation by Mycobacterium smegmatis on the surface of a liquid medium. Utilizing high-resolution two-dimensional gel electrophoresis in combination with mass spectrometry they **identified proteins involved in regulation of early stages of pellicle formation** by M. smegmatis. These proteins are similar to those participating in the maturation of older biofilms identified by others. Since biofilms are the source of many infections, they represent an excellent drug target. Therefore, understanding the principles of the early stages of biofilm formation might be very useful (Sochorová et al., 2014). The work was performed in collaboration with the Group of electron microscopy of the Institute of Microbiology.

We aimed the next study to clarify **the role of 6S RNA** in the **control of antibiotic biosynthesis** in Streptomyces coelicolor. Deletion of the ssrS gene from S. coelicolor affects the **growth rate** and influences the expression of several pathway-specific genes involved in **actinorhodin biosynthesis**. The complementation of the ΔssrS strain restored the wild-type growth rate and actinorhodin production. We concluded that **6S RNA contributes to expression of key genes for the biosynthesis of actinorhodin** (Mikulík et al., 2014).

Most of the experimental work was done at Institute of Microbiology. Part of the experiments was performed at First Faculty of Medicine, Charles University in Prague.

The group is currently engaged in main projects in the lab concerning molecular **signalling in Streptoccoci**.

The main focus of eukaryotic research team supervised by **T. Vomastek** has been on the **architecture and regulation of the ERK cascade**; in particular how is the ERK signaling, through the phosphorylation of functionally diverse substrates, implemented into specific biological response. We investigate **the role ERK** plays in the establishment of **migratory phenotype** using either epithelial cells induced to migrate or fibroblast cell establishing front-rear polarity upon integrin-mediated adhesion. In epithelial cells, ERK induce a series of morphological changes leading to the transition from static multicellular epithelium to motile single cell phenotype. We found that **ERK mediated activation of protease Calpain induces loss of apical-basal polarity whereas ERK mediated activation of kinase p90RSK induces cell autonomous migration.** Thus, by selection of different substrates ERK regulates two cellular subprograms that are both required for the gain of migratory phenotype. Identification of Calpain and p90RSK as potential ERK substrates involved in these processes suggest the hypothesis that the **establishment of migratory phenotype is achieved through control of functionally different ERK substrates**. In addition, it appears that there is hierarchy between these regulatory subprograms that permits their coordinated execution in time. These results were published in Cellular Signalling (Čáslavský et al., 2013). This work was entirely done at Institute of Microbiology. Complementary work with spreading cells revealed that **ERK is required for the disruption of radial symmetry of spreading cells** as ERK enables cells to form non-protruding cell rear. This process is crucial for defining cell's front and rear and represent prelude to cell migration. The symmetry breaking is achieved by the ERK mediated inhibition of p190RhoGAP at the prospective cell rear.The manuscript concerning the role in the formation of non-protruding cell rear has been recently submitted.

In a second project we are investigating **the role of nuclear movement on cell polarity**. The establishment of migration polarity, an essential step in the process of cell migration, requires precise spatiotemporal coordination of signaling pathways that ultimately create the typical asymmetrical profile of a polarized cell with nucleus located at the cell rear and the microtubule organizing centre (MTOC) positioned between the nucleus and the leading edge. We found that **cell polarization and directional migration requires the reorientation of the nucleus**. Nuclear reorientation is manifested as temporally restricted nuclear rotation that aligns the nuclear axis with the axis of cell migration. Nuclear reorientation is controlled by coordinated activity of lysophosphatidic acid mediated activation of GTPase Rho and the activation of integrin, FAK, Src and p190RhoGAP signaling pathway. Our data suggest that integrin activation within lamellipodia defines cell front and subsequent **FAK, Src and p190-RhoGAP signaling represents the polarity signal that induces reorientation of the nucleus** and thus promotes the establishment of front-rear polarity. These results were published in Journal of Molecular Biology (Maninová M et al., 2013). This manuscript is a result of collaboration with Drs. M.J. Weber, J. T. Parsons (University of Virginia, USA) and M.P. Iwanicki (Harvard University, USA). Another manuscript concenring nuclear movement has been published in Cell adhesion & migration (Maninová et al, 2014). This manuscript is a result of collaboration with Dr. M.P. Iwanicki (Harvard University, USA). The experimental work was entirely done at Institute of Microbiology, the collaborating members provided us with necessary materials and reagents. Recently we found that nuclear movement and cell polarization requires coordinated action of transverse actin arcs, perinuclear actin cap and dorsal stress fibers. The manuscript concerning the role of nuclear movement and cell polarity will bee submitted soon.

C) INTERNATIONAL COLLABORATIONS

We maintain active international research collaborations, many of them resulting in joint papers, with investigators such as:

Amir Aslam, University of Oxford, UK
Jesus Sanz, Universidad Alicante, Spain
Shahriar Mobashery, University Notre Dame, USA
Juan Hermoso, CSIC, Madrid, Spain
Virginie Molle, Universite Lyon, France
Jan-Willem Veening, Rijksuniversiteit Groningen, Netherlands
Orietta Massida, Universita di Cagliari, Italy
Waldemar Vollmer, Newcastle University, UK
M.P. Iwanicki, Harvard University, USA
M.J. Weber, J. T. Parsons, University of Virginia, USA

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Regulation of Gene Expression

My team counting on average 10 people (during the current evaluation period) is still rather young, formed mostly by very motivated and enthusiastic Diploma and PhD students, all of which have a tremendous share on our rather productive phase of existence.

In detail, in the past 5 years we remained focused on three major aspects of our research: 1) elucidation of molecular details of translation initiation in the model yeast *S. cerevisiae* and human cell lines; 2) solving the structure of the key translation initiation factors and determining their binding sites on the small ribosomal subunit; and 3) determining molecular requirements for the gene-specific regulatory mechanism called reinitiation. Along the way we have also become interested in 4) studies of translation termination and programmed stop codon read-through as well as examining the functional complementarity between ribosomal RNAs and mRNA's 5' and 3' UTRs.

Ad 1) We described functions of several subunits of multifunctional translation initiation factor eIF3 and its associated companions eIF1, eIF1A, eIF2 and eIF5 in general translation initiation. For example, we showed for the first time that two eIF3 subunits are directly involved in "scanning" the 5' untranslated region for the initiating AUG and proposed the mechanism of their action. Moreover, we established several key techniques that have enabled us, as the first group world-wide, to begin studying the role of all 13 subunits of mammalian eIF3 in general translation initiation in living cells using the RNAi approach. Our research covering this topic resulted in altogether 5 impacted publications with me featuring as a corresponding author on all of them except the last one on this list (Wagner et al., 2014, *Mol. Cell Biol.*, 34(16), 3041-52; Karasková et al., 2012, *J Biol Chem,* 287, 28420-28434; Cuchalová et al., 2010, *Mol Cell Biol*, 30, 4671-4686; Chiu et al., 2010, *Mol Cell Biol*, 30, 4415-4434; Nemoto et al., 2010, *J Biol Chem*., 285, 32200-12).

Ad 2) In collaboration with Peter Lukavsky (MRC-LMB, Cambridge) and Ralf Ficner (UNI of Goettingen) we solved crystal structures of three individual subunits of yeast eIF3 (or their interacting domains) and described where they contact the small ribosomal subunit and what is their functional contribution to the overall course of translation initiation (Khoshnevis et al, 2014, *Nucleic Acid Res.*, 42(6):4123-39; Herrmannová et al., 2012, *Nucleic Acid Res.*, 40(5), 2294-311; ElAntak et al., 2010, *J. Mol. Biol.*, 396, 1097-116). Our contribution on all these studies was 50% - genetic and biochemical analysis of the solved structures.

In addition to that, we mapped several critical intermolecular bridges between eIF3 and eIF4B and the small 40S ribosomal subunit and characterized their roles in translation initiation. This research produced altogether 3 impacted publications with me featuring as a corresponding author on all of them except the last one on the list (Kouba et al., 2012, *Nucleic Acid Res.*, 40(6), 2683-99; Kouba et al., 2012, *PLoS One*, 7, e40464; Walker et al., 2013, *RNA*, 19(2), 191-207).

Ad 3) We revealed what makes the small ribosomal subunit to stay bound to the mRNA after termination of translation of short upstream ORF (uORF) in order to resume scanning for the reinitiation of the small mRNA molecule but downstream. In particular, we described several *in cis* acting mRNA features preceding coding regions of short uORFs that interact with eIF3

and this way stabilize the 40S subunit on mRNA during the recycling phase of termination (Munzarová et al., 2011, *PLoS Genet.*, 7, e1002137). In addition to that, we explained for the first time why does the long-standing model gene of translation reinitiation, in yeast *GCN4*, contain 4 uORFs in its 5' UTR by postulating and evidencing the fail-safe regulatory mechanism of its very distinct mode of regulation (Gunišová et al., 2014, *Nucleic Acid Res.*, 42(9), 5880-93).

Ad 4) Being inspired by a hint of our foreign colleagues, we embarked on a risky business in trying to prove or disprove a notion that two translation initiation factors – namely eIF3 and HCR1 – control besides the initiation also the termination phase. This assumption turned out to be true and, in addition, we also showed that both of these factors may co-regulate another important regulatory process - programmed stop codon readthrough (Beznosková et al., 2013, *PLoS Genet.,* 9, e1003962). Finally, we revealed an evolutionary conserved pattern of 18S rRNA sequence complementarity to mRNA 5' UTRs and proposed that it modulates scanning of the 40S subunit through 5' UTRs of mRNAs (Pánek et al., 2013, *Nucleic Acid Res.*, 41(16), 7625-7634 – this is our in-house collaboration with Dr. Vohradsky).

Besides these achievements, two methodology papers and one small (von der Haar and Valášek, 2014, Fungal RNA Biology, Springer International Publishing, Switzerland, 2014) and one major (Valášek, 2012, *Curr Protein Pept Sci.*, 13, 305-330) reviews were published within the evaluation period.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Environmental Microbiology

The Laboratory of Environmental Microbiology consisted on December 31, 2014 of three senior researchers, the rest of the scientific staff being represented by postdoctoral fellows (3), PhD students and MSc students. Two of the postdoctoral fellows joined during the last three years being selected among candidates that responded to the position offer, the third postdoctoral fellow is a former PhD student. The group consists of researchers from Czech Republic, Slovakia, Spain and Canada.

During 2010-2014, the field of microbial ecology faced an unprecedented development thanks to several methodological advances, most importantly the rapid development of high-throughput sequencing methods, but also steps that may look as relatively minor, such as the ability to extract RNA and proteins from environmental matrices reliably and in sufficient yields, or advances in proteomics. This development was reflected by the research focus of the group which extended its interest in the biochemistry and enzymology of the environment to the analysis of microbial communities and, most importantly, made the use of the combination of these approaches in the focused research concerning the functioning of soil ecosystems, especially those of the forest ecosystems. Due to the rapid establishment of novel methods – the group was among the first that used long-read next-generation-sequencing (NGS) platform to explore fungal communities (Baldrian et al. 2012), as well as the first to use NGS in combination with stable isotope probing (Stursova 2012), an NGS-based survey of the expressed pool of a gene in the environment (Baldrian et al. 2012) or obtained one of the first comprehensive metatranscriptomic dataset from forest soils (Zifcakova et al., submitted) – the laboratory belongs among the leaders in the functional analysis of soil microbial communities. Considering subjects, the group has a large portfolio that covers the following, closely related topics of biochemistry of natural environments, exploration of factors shaping composition, function, and dynamics of microbial communities in soils, involvement of fungi in decomposition, and plant-microbe interactions. Since 2010 an effort was made to extend the original focus that more considered fungal processes to those ecosystem processes, performed by bacteria. Two new postdoctoral fellows, Ruben Lopez Mondejar and Salvador Llado were able to generate first interesting results in this field along with other group members and may develop into potential leaders of a group devoted to this topic. Also, the collaboration with Cardiff University and Yale University started the collaborative research on the interactions of fungi with invertebrates and the importance of tritrophic interactions on ecosystem processes such as decomposition.

Biochemistry of natural environments

The environmental factors, such as, most importantly, soil chemistry, vegetation and land use were explored and demonstrated to have different levels of importance for soil processes, determined as enzyme activity (Stursova and Baldrian 2011). The extension of these studies showed, that the plant-induced effects on the chemical properties in litter and soil make the decomposition processes highly affected by the identity of the dominant tree (Voriskova et al. 2011, Snajdr et al. 2013). The paper of Eichlerova et al. (2012) demonstrated

that the activity of enzymes in soils are not only determined by the rates of their production but also by the kinetic parameters of enzymes in each respective environment and showed that reliable biochemical estimations of enzyme activities can be only obtained with cautiously selected assays. Biochemistry of soils was the subject of review papers on the enzymology of forest soils and determinants of enzyme activities at various spatial scales (Baldrian and Stursova 2011, Baldrian 2014) that extend the observations of individual studies to the ecosystem level.

Factors shaping the composition, function and dynamics of microbial communities in soils

In forest ecosystems, vertical stratification of organic matter and physico-chemical properties of soil is of high importance. Our research demonstrated that these differences in soil properties among soil horizons are reflected by profound differences in community composition of fungi as well as bacteria (Baldrian et al. 2014, Voriskova et al. 2014, Lopez Mondejar et al. 2014). Moreover, we have demonstrated that the seasonal differences in various soil horizons, such as the occurrence of fresh litter or rhizodeposition of C during vegetation season are responsible for the changes of microbial community composition along the year (Voriskova et al. 2014, Lopez Mondejar et al. in press). These changes in microbial community composition have important consequences in the seasonality of microbe-catalysed processes during the year (Baldrian et al. 2013, Voriskova et al. 2014).

We have also studied the decomposition of litter with a complex array of analytical tools to show that three phases of decomposition can be identified with respect to changes of soil chemistry (Snajdr et al. 2011). The succession of fungi on decomposing litter was demonstrated to be much more dynamic than predicted by previous models with a fast turnover of community composition (Voriskova and Baldrian 2013), however, the rate of successive changes corresponds to the overall turnover rate of specific litter types (Koukol et al. 2012, Voriskova et al. 2013, Hanackova et al. 2015)

In addition to seasonal dynamics of ecosystems, succession of microorganisms and changes of the microbial processes can be also observed on a longer time scale that considers ecosystem development: dramatic changes of microbial communities and decomposition are associated with a primary succession on post-mining barren soils (Urbanová et al. 2011, 2014) as well as during forest dieback and recovery after a bark-beetle invasion which represents a dramatic event with profound alteration of a complex of ecosystem processes (Stursova et al. 2014).

The study of the function of microbes in the soil using RNA sequencing allowed us, for the firs time, to estimate the diversity of transcripts of an eukaryotic gene – cbhI cellulase, and gave the answer that catalysis of cellulose decomposition is highly redundant, being performed by tens of fungi in the same time (Baldrian et al. 2012). Metatranscriptome analysis later extended this observation to a wide range of fungal and bacterial genes, often expressed in hundreds of variants and clearly showing the high functional redundance of the soil environment (Zifcakova et al. submitted). Importantly, in addition to nutrient dynamics, interactions between fungi were identified as potential important factors driving enzyme production and, consequently, decomposition (Hiscox et al. 2010, Šnajdr et al. 2011).

Involvement of fungi in decomposition

The importace of fungi in decomposition processes was well known since long. Using systematic screening of environmentally-relevant groups of decomposer fungi, we have defind the differences in decomposition potential of various ecophysiological and taxonomic groups with saprotrophic cord-forming basidiomycetes being the most efficient decomposers

of polysaccharides and lignin (Snajdr et al. 2010, Baldrian et al. 2011, Eichlerova et al. 2015, Koukol and Baldrian 2012) showing that genome content only offers limited information on fungal decomposition potential (Eichlerova et al. 2012). Interestingly, Stable-Isotope-Probing (SIP) and screening of isolates indicated that nonbasidiomycetous fungi are also highly involved in cellulose decomposition and are likely more important decomposers as previously expected (Zifcakova et al. 2011, Stursova et al. 2012).

Ecology of bacteria in forest soils

Traditionally, the decomposition abilities of bacteria were assumed to be of much lower importance than that of fungi. This assumption was changed by our finding that bacterial obtain more C from decomposing cellulose in forest litter than fungi and are multiple, so far unknown taxa are likely highly active in cellulose decomposition (Stursova et al. 2012). This finding promoted our interest in the involvement of bacteria in decomposition and in their ecology in general. Screening of soil Actinobacteria showed that some taxa are indeed able to grow on complex lignocellulose substrates and to transform efficiently polasaccharides and phenolics (Vetrovsky et al. 2014). Further experiments showed that abundant bacterial taxa (such as several members of the Acidobacteria) are potent producers of polysaccharide hydrolases and, considering their abundance in soils, are likely highly important in thr turnover of organic matter in forest ecosystems (Llado et al. submitted, Lopez Mondejar et al. 2015). In the former paper, we were the first to report about the metabolic potentials and enzyme production of bacteria belonging to the most abundant in soil. The ecology of important bacteria is now further explored using single-cell genomics.

Interaction of microorganisms and plants

Trees as dominant primary producers in forest ecosystems were repeatedly demonstrated to affect the soil microbiome and its functioning. We have used the opportunity to compare replicated stands of various trees developed on the same, initially barren substrate, to quantify the effects of trees on soil microbes in a defined system and at a resolution that was not available before. Tree identity was indicated as the most important driving factor of the composition of soil fungal and bacterial communities in forest litter and soil, fungal communities being significantly more tree-specific (Urbanova et al. 2015). These tree effects are partly due to differences in litter and soil chemical properties (Snajdr et al. 2013) and result in differences in ecosystem productivity and organic matter turnover, reflected as the activity of decomposition-related enzymes (Snajdr et al. 2013). Partly, the tree effects on microbes are indirectly mediated by soil fauna (Frouz et al. 2013)

Interactions of fungi with invertebrates and its impact on environmental processes

The topic of interactions between fungi and invertebrates were the result of collaboration with the Cardiff University. Invertebrate grazing was known to be a quantitatively important mode of predation. Our joint papers with CU demonstrated that grazing affects decomposition by regulating fungal production of enzymes (Crowther et al. 2011) and that grazing pressure can result in the replacement of the grazed taxa by others (Crowther et al. 2013). The contribution of fungi and invertebrates to decomposition was the topic of a study headed by Yale University where our group participated. The results of a comprehensive experiment with decomposition under various climatic contexts indicates that climate is not the most important driver of decomposition as previously thought (Bradford et al. 2014).

Methodological advancements

In addition to the struggle to use the most up-to-date methodology in research, our group has significantly contributed to the development of novel methods. In the paper of Baldrian et al. (2012) we have developed a novel method for RNA extraction from soils that allowed us to obtain sufficient amounts of RNA from soils with low pH and high organic matter content. Moreover, the paper demonstrated the power of amplicon analysis of a functional gene for the exploration of functional diversity in the environment. Later, the efforts to analyze microbial function were successfully demonstrated by the analysis of the first forest soil metatranscriptome with sufficient replication of samples in time and space with a prediction of several millions of protein sequences and showing the feasibility of metatranscriptomics in forest litter and soil (Zifcakova et al. submitted). The fact that our group entered the field of NGS among the first made it necessary to work on the improvement of methods for amplicon analysis. This resulted in the development of a software tool for high-throughput amplicon analysis and associated workflows (Vetrovsky and Baldrian 2013a). We have also re-evaluated the importance of 16S diversity and abundance in bacterial genomes by showing how these factors may affect the results of bacterial community analysis. In the paper of Vetrovsky and Baldrian (2013b) we have proposed and tested the method of prediction of bacterial genome counts based on 16S sequence counts. The comparison of methods for fungal biomass determination was the subject of a comparative study leading to the assessment of the potential and limitations of the common methods (Baldrian et al. 2013). Methods for laccase activity measurements were critically assessed and evaluated by Eichlerova et al. (2012) and the method of analysis of enzyme activity variation in space was developed with the highest resolution achieved so far (2.5 mm), applicable to various environmental samples (Baldrian and Vetrovsky 2012). The impact of these methodological advances is demonstrated by the high citation rates of the papers of Baldrian et al. (2012, 2013) and Vetrovsky and Baldrian (2013a,b).

International collaboration and visibility of the team

In 2010-2015, the bulk of the research was represented by projects that originated from the resarchers of the laboratory, but certain subjects were explored in collaboration with Czech as well as foreign scientific institutions. The international collaboration was focused on the exploration of interspecific interactions of fungi () and the effects of grazing on fungi (), both jointly studied with Cardiff University (Prof. Lynne Boddy). The collaboration with Yale University explored the drivers of decomposition and responses to global change (Bradford et al. 2014). The assembly and analysis of metatranscriptome data was performed in a collaboration with Dr. Adina C. Howe (Iowa State University) who provided training and access to large computing clusters. Extensive collaboration was maintained with Prof. Katharina Riedel (University of Greifswald) in the field of proteomics and metaproteomics of microbial cultures, soil and litter (results will be submitted for publication this year). Last but not least, the laboratory was involved in the ITN Marie Curie project „TRAINBIODIVERSE - Training for functional soil microorganism biodiversity“ in a collaboration with eight European high-rank universities and hosted their students/sent their students for stays. Ad-hoc collaborations were maintained with persons or groups from within COST Actions where the laboratory members participated.

The international visibility of the group was high, considering the reflection in citations. There were 68 IF papers published by the group in 2010-2014 which were cited (19.4.2015) 697 times according to wos, with an h-index of 16 (sixteen papers cited more than

16 times). The most cited papers received the following counts of citations: Baldrian et al. 2012 – 81, Snajdr et al. 2011 – 44, Baldrian et al. 2011 – 38, Vetrovsky et al. 2013 – 36, Stursova et al. 2012 – 34, several papers belonged to the most cited in the respective journals where they were published. The visibility was also achieved by active participation in international conferences, including several invited lectures (Petr Baldrian), such as, for example, at the Ecological Society of America Annual Conference in Austin, 2012, Soil Metagenomics Conference, Braunschweig, 2013, International Mycological Congress (IMC) 2014, Bangkok, International Symposium for Microbial Ecology (ISME), 2014, Seoul. Petr Baldrian was the head of the Organizing Committee of a newly established conference „Ecology of Soil Microorganisms" that took place in Prague in 2011 and he will also chair the same conference to be held in 2015. P. Baldrian was also the convener of topical sessions on Decomposition in Forest Ecosystems and Fungal Ecology at IMC in Bangkok and at ISME in Seoul. He is also the International Society for Microbial Ecology Ambassador for the Czech Republic. Several papers were published in journals on popular science (in Czech).

Most important projects with PI or co-PI from the laboratory:

FP7-PEOPLE-2011-ITN (Marie Curie Actions-Initial Training Networks) - TRAINBIODIVERSE—Training for functional soil microorganism biodiversity (co-PI Baldrian) 2012-2015
Czech Science Foundation - GAP504/12/0709 - Basidiomycetous fungi: exploration and preservation of biodiversity and involvement in environmental decomposition processes (PI Eichlerová) 2012-2016
Czech Science Foundation - GPP504/12/P107 - Turnover of fungal biomass in forest soil and identification of the structure and function of microbial community participating in its degradation (PI Brabcová) 2012-2015
Czech Science Foundation - GA13-27454S - Deadwood decomposition dynamics in natural temperate forests (co-PI Baldrian) 2013-2017
Czech Science Foundation - GP14-09040P - Ecology of the dominant bacterial taxa in the Picea abies forest soil (PI Lladó Fernández) 2014-2015
Czech Science Foundation - GA526/08/0751 - Soil processes and microbial communities related to C and N cycles during spruce forest recovery after bark beetle outbreak (PI Baldrian) 2008-2012
Czech Science Foundation - GAP504/12/1288 - The role of leaf functional traits in soil organic matter accumulation during primary sucession (co-PI Baldrian) 2012-2016
Czech Science Foundation - GA13-06763S - Fungi in forest soil and litter: biogeography and ecology at a regional scale (PI Baldrian) 2013-2017
Ministry of Education, Youth and Sports of the Czech Republic - LC06066 – Center for Environmental Microbiology (PI Nerud) 2006-2011

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Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Environmental Biotechnology

The Laboratory of Environmental Biotechnology is focused on research topics in the area of microbial decomposition of organic pollutants in the environment. The research includes basic science approaches and the study of microbial mechanisms for the degradation of organic pollutants. However, the processes are also studied from the application perspective in order to utilize the decomposition processes to clean up polluted sites using specific microorganisms. The research is prevalently interdisciplinary and, besides microbiology, other main scientific fields are biochemistry, toxicology, environmental and analytical chemistry and molecular biology. Within the period 2010-2014, members of the team published 60 articles in impacted journals and successfully submitted 3 patents.

The research areas of the above cited period can be divided according to several perspectives. From the basic research point of view, the main aspects under study were interactions of microorganism with respect to their decomposition properties and anthropogenic organic pollutants, including their general fate in the environment and ecotoxicology. The applied research was mainly dedicated to the development and optimization of bioremediation technologies, prevalently represented by the method of mycoremediation and other decontamination methods. Due to different properties and types of the contaminated environmental matrices, the research focus can also be simply divided into "classical" persistent organic pollutants and new emerging micropollutants (e.g. pharmaceuticals, personal-care products and endocrine disrupting compounds).

One of the main subjects was the study of polychlorinated biphenyls (PCBs) degradation by ligninolytic fungi. PCBs belong amongst persistent organic pollutants because of their environmental recalcitrance and ecotoxicity. Although their production was prohibited a long time ago, they still persist in the environment and represent a serious environmental problem. One of the few available techniques to decontaminate PCB-polluted matrices consists in thermal physico-chemical methods, that represent technically and financially demanding approaches. Alternatively, bioremediation has attracted greater public interest as an effective and economically feasible strategy for the removal of various pollutants. PCBs can be degraded by aerobic bacteria *via* several pathways, the most important and widely studied of which is a co-metabolic process performed by biphenyl catabolic enzymes. Unfortunately, this pathway leads to the formation of chlorobenzoic acids (CBAs), which tend to accumulate as dead-end products. Moreover CBAs inhibit further biodegradation of PCBs. Therefore, it is worth looking for other microorganisms that are able to degrade PCBs.
Ligninolytic fungi (LF), with their extracellular, low-substrate-specificity enzymes, represent a promising alternative for the biodegradation of various aromatic pollutants (Cajthaml, T., Svobodová, K., 2011. Microbial Degradation of Xenobiotics. Springer-Verlag Berlin Heidelberg). In contrast to a large number of articles dealing with bacterial degradation of PCB, only a few papers have been published describing the degradation potential of fungi towards these compounds. The nature of PCB-biotransformation products and the overall contaminants breakdown mechanisms are yet poorly understood, this representing the major

limiting factors for the development of robust bioremediation applications. The main aim of our contributions was to select promising ligninolytic fungal strains for degradation of a technical mixture of PCBs and to clarify the respective degradative mechanism.
The selection of fungi was performed under model laboratory conditions using technical mixtures and also individual congeners of PCBs. Our preliminary data regarding the detection of transformation products revealed that CBAs are produced from PCBs also by this group of fungi. Due to a certain risk linked with this phenomenon we performed similar incubations with CBAs isomers. The results of this study showed that the tested fungi are generally very efficient in the degradation of CBAs. **To the best of our knowledge, this was the first paper providing a general description of the ability of fungi to biodegrade CBAs. Such capability was proved under model laboratory conditions (i.e. axenic fungal cultures) and also in a soil contaminated by chlorobenzoates. Numerous new metabolites were detected and characterized, including ring-fission products, and a novel pathway has been suggested.** The study represents a part of 1 MSc thesis (Muzikář et al., 2011, Journal of Hazardous Materials, 196, 386-394). After this, we continued studying the degradation of PCBs and the data from this work proved the ability of fungi to degrade PCBs with different level of chlorination, namely from mono- to hexa-chlorinated congeners. Numerous new PCB metabolites were identified and a completely novel transformation pathway was proposed. **Together with the previous work we provided evidence that the fungi are able to degrade further the main metabolites on the pathway (CBAs), more rapidly and simultaneously with PCBs. The most efficient fungus was *Pleurotus ostreatus* (oyster mushroom) that was able to decompose more than 99% of the added PCBs, this being accompanied by a decrease in samples ecotoxicity.** The study represents a part of 1 MSc and 1 PhD thesis and the whole work was performed within the team of the laboratory (Čvančarová et al., 2012, Chemosphere, 88, 1317‑1323).
As next step, we focused on the PCB transformation mechanism. By carrying out numerous experiments using crude culture extracts and purified and semi-purified ligninolytic enzymes we demonstrated that these enzymes are not able to perform the primary attack of PCB molecules, this representing a novelty with respect to the scientific literature. **Regarding the hydroxylated structures of PCBs and CBAs that we detected throughout incubations, we further focused on the possible participation of cytochrome P-450 and this enzymatic system was finally proved to initiate the oxidation of CBAs. To the best of our knowledge, this was the first study evidencing the degradation of chlorinated pollutants by fungal cytochrome P-450 under in vitro conditions** (Stella et al., 2013, Journal of Hazardous Materials, 260, 975-983). The study represents a part of 1 MSc and 1 PhD thesis of a student co-supervised with the University of Tuscia, Viterbo, Italy. The whole work was carried out within the team of the laboratory and two Italian colleagues participated in the manuscript preparation. The study dealing with PCBs transformation by P-450 is still in progress.
In the frame of these studies we had to develop and optimize several novel analytical methods, in order to be able to perform the degradation studies (e.g. Křesinová et al., 2011, Talanta 84, 1141-1147; Křesinová et al., 2014, International Journal of Environmental Analytical Chemistry, 94, 822-836).

Another subject under study at the Laboratory of Environmental Biotechnology represents fungal degradation of polycyclic aromatic hydrocarbons (PAHs) and phenomena related to the subject.
A novel strain of ligninolytic fungi (*Lentinus tigrinus)* was described as highly efficient in the decomposition of PAHs. The results showed extremely high degradation efficiency which reached 97% under the conditions of our study. A number of transformation

products were identified and the participation of specific fungal enzymes was also described. (Covino et al., 2010, Bioresource Technology, 101, 3004-3012). The work was partially performed at the Italian university (cultivation of the fungus) and in our laboratory (all the analyses including purification and characterization of the enzymes). The same fungal strain was tested for the degradation of PAHs in real contaminated matrices (soil, railway cross-ties) originating from a wood treatment plant. Several aspects of the decontamination such as ecotoxicity and pollutants bioavailability were monitored. **Although PAH degradation was significantly correlated with their intrinsic bioavailability, the breakdown of certain PAHs proceeded well above their respective bioavailability threshold.** The work was completely performed at our laboratory and the Italian supervisors of our common student assisted the preparation of a manuscript (Covino et al., 2010, Journal of Hazardous Materials, 183, 669-676). Both the studies represent a main part of 1 PhD thesis of the student.

In the frame of this subject we optimized and developed a novel method based on sequential mild extraction with supercritical fluids, in order to predict bioavalability (bioaccessibility) of PAHs in real contaminated soil, and the instrument together with the method have been patented. (CZ304286/PV 2013-294 A method of extraction of polycyclic aromatic hydrocarbons (PAHs) form soil samples and the respective instrumentation). This work was almost fully carried out within the team of the laboratory, with an assistance of a team from Dekonta a.s. company (sample delivery, patent application preparation).

A method based on CO_2 supercritical fluid extraction was applied in order to prove or to decline a hypothesis of influence of the bioaccessible fraction of polycyclic aromatic hydrocarbons on the ecotoxicity of historically contaminated soils. **Despite the extensive scientific communication about the subject, this is to our knowledge the first contribution providing evidence for the relationships among bioaccessibility of pollutants and ecotoxicity of contaminated soils.** The study was completely performed at our laboratory (Čvančarová et al., 2013, Journal of Hazardous Materials, 254, 116-124). The study represents a part of 1 PhD thesis.

During the monitored period, we developed common projects with several private companies specialized in sites reclamation. In one of our works, a completely new decontamination approach through bioaugmentation was developed. Generally, classical bacterial bioaugmentation methods rely on massive pre-cultivation of degradative strains from culture-collection in common nutrient media and this inoculum is consequently spread onto contaminated sites. Such culture media, however, can not contain the target contaminants and as a consequence the strains usually loose their degrading ability during this step. Therefore a certain period of pre-adaptation of the inoculum is necessary prior to mixing with the contaminated matrix. Our novel approach is based on sequential, stepwise mixing of an inoculated contaminated soil with larger amount (10 times higher) of the non-inoculated soil originating from the contaminated site. We successfully tested this approach under laboratory conditions using a real PAHs contaminated soil in the amount of several metric tonnes. 8 bacterial strains previously isolated from other contaminated sites and provided by Dekonta a.s. were used for soil decontamination tests. **The degradation of PAHs reached 70% in the real contaminated soil during the second step of the inoculation and 80 % in the case of the artificially contaminated soil within a 4 months period. In order to prove the viability of the used strains, DNA was isolated from the samples during the inoculation process and the adaptation of the bacterial strains was evaluated using 454 sequencing of 16S rDNA. The method was successfully patented and it is currently used by the company (Patent: CZ304378/PV 2013-240).** The laboratory work and all the

chemical/microbiological analyses were performed at our laboratory. The pilot test manipulation was done by the cooperating company. The study represents a part of 1 master thesis.

Regarding the subject of the new emerging pollutants, we performed several ecotoxicology and degradation studies with pharmaceuticals and typical estrogen-like endocrine disruptors.
Synthetic estrogen 17α-ethinylestradiol (EE2) is widely used in oral contraceptive pills. Trace concentrations (from ng to μg per liter) of EE2 and its conjugates, have been detected in wastewater treatment plants, their effluents, surface and groundwaters and also in drinking water reservoirs. This compound is one of the strongest known estrogens that is unfortunately rather resistant to biological decomposition. Therefore we performed a mechanistic study of EE2 biodegradation by ligninolytic fungi that was designed in order to clarify which enzyme machinery participate in the biotransformation mechanism.
A set of in vitro experiments using various cellular fractions and enzyme suspensions from *Pleurotus ostreatus* has been performed and the results showed that EE2 was degraded by isolated laccase (about 90% within 24 h). The degradation was also tested with concentrated extracellular liquid where degradation reached 50% mainly due to laccase activity; however, after supplementation of the medium with H_2O_2 and Mn^{2+} EE2 degradation reached 100%, due to residual manganese-dependent peroxidase activity (40 times lower than Lac). Moreover, the intracellular fraction and also laccase-like activity associated with fungal mycelium were found to be efficient in the degradation too. In addition, isolated microsomal proteins appeared to be also involved in the process. The degradation was completely suppressed in the presence of cytochrome P-450 inhibitors, piperonylbutoxide and carbon monoxide, thus indicating a role of this monooxygenase system in the initial degradation process. Attention was also paid to monitoring of changes in the estrogenic activity during these particular in vitro experiments and the most effective systems in term of EE2 removal and estrogenic activity decrease where those with ligninolytic enzymes. Several novel metabolites of EE2 were detected using different chromatographic method with mass spectrometric techniques (LC-MS, GC-MS) including also [^{13}C]-labeled substrates. The results document the simultaneous involvement of different enzymatic mechanisms in the degradation of EE2 by *P. ostreatus*, i.e. the ligninolytic system and the eukaryotic intracellular cytochromes P-450.
To the best of our knowledge, this is the first article describing metabolites of 17 α-ethinylestradiol by a ligninolytic fungus and characterizing the specific enzymes involved in EE2 degradation, such as ligninolytic enzymes, intracellular fraction and cytochrome P-450. The work was mostly carried out at our laboratory while only special analyses (e.g. MS/MS experiments and utilization of HPLC-MS) were performed at the cooperating laboratory. The study is included in the context of 1 PhD thesis (Křesinová et al., 2012, Environmental Science & Technology, 46(24), 13377‑13385).

Another study was aimed at clarifying the decomposition mechanism of a fluorinated antibiotic compound – i.e. flumequine (FLU). Flumequine, a fluoroquinolone antibiotic, is applied preferably in veterinary medicine, for stock breeding and treatment of aquacultures. Development of drug resistance is a matter of general concern when antibiotics such as flumequine occur in the environment. Although the acute toxicity of flumequine toward aquatic organisms is relatively low, recent studies confirmed that flumequine can be bioaccumulated and exhibits genotoxic and carcinogenic properties. As reported previously, FLU is not degraded in water/sediment slurries from aquacultural ponds under exclusion of light and, photolysis is emphasized as the main abiotic transformation process of flumequine in water. Our contribution to this research subject was to comparatively assess biodegradation

potential of various fungi, to elucidate the chemical structure of FLU metabolite and to monitor potential toxicological outcomes. Several fungal strains were compared for their ability to decompose this recalcitrant compound and some of them transformed more than 90% within 3 days. Analyses of the metabolites by liquid chromatography−mass spectrometry suggest different transformation pathways for the different fungal strains. Structure proposals were elaborated for 8 metabolites. 7-Hydroxy-flumequine and flumequine ethyl ester were identified as common metabolites produced by all ligninolytic fungi. Residual antibacterial activity of the metabolite mixtures was tested using gram-positive and gram-negative bacteria. While for the less efficient fungal cultures the antibacterial activities corresponded to the residual concentrations of flumequine, a remarkable antibacterial activity remained in the cultures of the fungus *Dichomitus squalens*, although FLU was transformed to an extent higher than 90%. Obviously, transformation products with a high antibacterial activity were formed during incubation with this fungal strain. **A novel microbial degradation pathway has been proposed, the residual antimicrobial activity was determined and the results exhibited that transformation of FLU is not necessarily linked to the loss of antibacterial activity. This emphasize the need of monitoring of residual biological activity after treatments in order to assess the environmental tolerance and potential risks associated to reclamation processes, even in the case of efficient or complete removal of the target substance.**
The extraction of fungal cultures and ecotoxicological assays were performed at our laboratory. The intermediate structures were suggested by the group of collaborators. The study represents a part of 1 PhD thesis (Čvančarová et al., 2013, Environmental Science & Technology, 47, 14128-14136).

Over the last few years, there has been a great interest in using nano-scale zero-valent iron for various reductive-remediation applications. Despite the efficiency of ZVI and especially nano-scale ZVI (nZVI) in the reduction of hexavalent chromium (Cr(VI)) and other pollutants is well documented, only few works focused on its ecotoxicity towards the indigenous organisms in the treated soil. Moreover, it is increasingly evident that classical ecotoxicological tests are not applicable for nanomaterials.
The aim of our contribution was to perform a pilot-scale in-situ remediation experiment in the saturated zone of a historically Cr(VI)-contaminated site using commercially available nZVI. Also, the effects of nZVI application on indigenous bacteria and groundwater toxicity were evaluated. The site was monitored before and after nZVI application by means of microbial cultivation tests, phospholipids fatty acid analysis (PLFA) and toxicological test with *Vibrio fischeri*. The test with *V. fisch*eri did not show any changes in toxicity of the groundwater after the nZVI application and no significant changes in cultivable psychrophilic bacteria densities nor in PLFA concentrations were observed during the course of the remediation experiment. **However, PLFA of soil samples revealed that the application of nZVI stimulated significantly growth of Gram-positive bacteria. Multidimensional statistical analysis was applied to the PLFA results in order to explain how Cr(VI) reduction and presence of Fe influenced the indigenous populations. The results clearly indicated a negative correlation between Cr concentrations and the autochthonous microbiota before the nZVI application and a significant positive correlation between bacteria and concentration of Fe after the nZVI application.** To the best of our knowledge, this is the first study describing the results of a real pilo-scalet in situ application of nano zerovalent iron (nZVI) in saturated soil, accompanied by an ecotoxicological monitoring of the process using a cultivation independent approach, i.e. phospholipid fatty acid (PLFA) analysis. The in-situ works including drilling and sample collections were performed by Dekonta a.s. and Enacon

s.r.o. companies. Our team performed all the PLFA analyses, interpreted the results and prepared the article (Němeček et al., Science of the Total Environment, 485, 739-747).

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Fungal Biology

The first main output of the group within the evaluated period was gaining insights into the distribution and ecology of summer truffle (*Tuber aestivum*) within Czech Republic. By using novel molecular detection and quantification tools (Gryndler et al 2011 FEMS Microbiol Lett 318:84-91; Gryndler et al 2013 Mycorrhiza 23: 341-348) we showed that, cross to earlier beliefs, substantial amounts of truffle mycelium is present also in older roots of the host trees and that the truffle also colonizes many other plants species, previously thought to be non-hosts (Gryndler et al 2013 Mycorrhiza 23: 341-348, Gryndler et al 2014 Mycorrhiza 24: 603-610). By using the molecular detection tools on a broad range of field sample we uncovered several new and prospective truffle localities within the Czech Republic (publication pending). We also demonstrated that abundance of truffle mycelium along an environmental transect correlates with abundance of certain soil prokaryotes, with possible consequences for the interactions of truffle mycelium with soil resources (Streiblová et al 2012 FEMS Microbiol Ecol 80:1-8, Gryndler et al 2013 Environ Microbiol Rep 5: 346-352). One of the main consolidating papers on the growth rates of truffle mycelium in soil throughout a year is still pending publication (submitted to Soil Biol Biochem in 2014).

Second, in collaboration with other groups based at the Nuclear Physics Institute and at the University of Chemistry and Technology in Prague, we significantly (25-30% of the research efforts) contributed to uncovering the identity and heavy metal (Ag, Au, U) bioaccumulation potential of macrofungi with different life history strategies (mycorrhizal or saprotrophic; Borovička at al 2010 Sci Total Environ 408: 2733-2744; Borovička et al 2010 Soil Biol Biochem 42:83-91; Gryndler et al 2012 Biometals 25: 987-993; Borovička et al 2014 J Hazard Mater 280: 79-88). The great bioaccumulation potential of the saprotrophic fungi recorded in the fields has attracted further scientific interest in (and funding towards) uncovering the mechanisms of uptake and storage of the heavy metals by the fungi (Osobová et al 2011 New Phytol 190: 916-626), where our team currently contributes the microbiology and molecular-genetics expertises.

Third, a newly (2012) established research focus on interactions of arbuscular mycorrhizal (AM) fungi with their soil environment has been developing within the last 3 years, yielding a number of novel and broadly valid insights into the AM community assembly rules including geography structuring (Hart et al 2013 Symbiosis 59: 47-56; Thonar et al 2014 Mol Ecol 23: 733-746; Jansa et al 2014 Mol Ecol 23: 2118-2135), interactions with soil saprotrophic microorganisms and soil fauna (Duhamel et al 2013 Ecology 94: 2019-2029; Jansa et al 2013 Front Plant Sci 4: 134) and trading of mineral nutrients for carbon at the symbiotic interface between the plant and the AM fungus (Konvalinková et al 2015 Front Plant Sci 6:65). Establishment of this research line necessitated major changes in the lab organization and equipment including acquisition of a new mass spectrometer to follow up the fluxes of carbon and nitrogen between plants, rhizobia, AM fungi and the soil. Some of the previously published papers relied heavily on tight international collaborations (up to 70% contribution by

the foreign partners into the above papers). More papers with data obtained solely in our lab are currently in the pipeline (submitted and/or revised).
Team of the lab 143 is well balanced with respect to the age structure, with important share of young researchers including PhD students and a sufficient number of experienced senior researchers and technicians. The team recently underwent significant enlargement (as of 2012), now refocusing from ectomycorrhizal and soil fungal research to the arbuscular mycorrhizal symbiosis and carbon fluxing between plants and soil. The team greatly benefits from long-established molecular expertise and is gradually adding stable isotope tracing (^{13}C, ^{15}N, ^{18}O) as another core approach to its method repertoire, concentrating mainly on model experiments under glasshouse conditions, and addressing interactions within mycorrhizal fungal communities, soil heterogeneity, organic nutrients and interplay between mycorrhizal hyphae and soil saprobes. Our team is heavily involved in collaborative research with other groups both in the Czech Republic (J. Borovička, P. Kotrba, P. Šmilauer) and abroad (T. Kiers, E. Frossard, H. Gamper, A. Schnepf, E. Verbruggen, C. Wagg).

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Functional Cytology

Laboratory team has in average 3 members and 2 PhD students. Age structure of the team: Pavla Binarova senior researcher, head of laboratory, 60-65, Jindrich Volc senior researcher, 65-70, 0.3 part-time is covered from the project, Jana Chumova PhD, advanced postdoctoral position, 35-40, carrier brake 2010-2015 (maternity leave with two children), she will return in May 2015, PhD student Lucie Kohoutova, 30-35, (her thesis submitted December 2014), PhD student Hana Kourova, 25-30 and Gabriela Kocarova Technician 35-40.
In average 40 to 50% of our personal costs were covered from external research funds. Pavla Binarova was PI of 2 projects and co-PI of 3 projects from Czech grant agencies (2010-2014)

Our research is focused to molecular biology of cytoskeleton specifically to gamma-tubulin and its protein interactions and functions. In addition to study of acentrosomal pathway of microtubule nucleation we aim to elucidate functions of gamma-tubulin in cell division and its still enigmatic functions in the nuclei.
The team in spite of small size occupies a rather noticeable position in the field. The success of the team is based on continuity, consistency of the research, and personal qualities and complementary skills of the team members. The team is consolidated and achieved good international recognition and visibility. We collaborated with I. Meskiene Max Perutz Laboratories Univ. of Viena (MSMT Mobility Czech Austria 7AMB13AT013 2013-2013 Cellular role of MAP kinase signalling cacscade in plants – function in regulation of nucleation and organization of microtubular cytoskeleton), with T. Mészáros, Dept. of Medical Chemistry, Molecular Biology Semmelweis University Budapest (collaborative project OTKA NN 111085 Posttranslational regulation of γ-tubulin complex in plant microtubule nucleation and cell cycle control 2014-04-01- 2017), and with G. Daniel Dept. Forest Products/Wood Science, SLU, Uppsala, (project FORMAS 2011-416, Copper-tolerant wood degrading fungi: Their decay potential towards Cu-based wood protection systems and molecular mechanisms of Cu-detoxification 2012-2014).
We further collaborated with Institute of Experimental Botany (P. Binarova had her group there in 2010-2011), and our collaboration with proteomics and electron microscopy units within home institution enabled us to apply complementary approaches in our research.

We showed previously that compare to animal cells, where gamma-tubulin is predominantly present in centrosomes, plant gamma-tubulin is a more abundant protein distributed throughout acentrosomal cells. The plant cells thus provide a suitable model for the study of non-centrosomal cellular functions of gamma-tubulin that are present and currently intensively explored in all eukaryotes.

(1) We found that **AtAurora1 (Arabidopsis homologue of Aurora A) regulatory signalling together with AtTPX2 and gamma-tubulin are critical to spatio-temporal regulation of acentrosomal microtubule organization. We demonstrated for the first time that plant Aurora kinases are required for controlling the switch from cell proliferation to differentiation and endoreduplication.** We performed biochemical analyses, protein interaction and colocalization studies and cloning of AtAurora1-GFP with native promotor, manuscript was written and corresponded from our laboratory. MS analyses were performed by P.Halada (IM). Research on Aurora kinases (cloning, transformation, microscropical and phenotype analyses) was performed in collaboration with IEB under coordination of P.Binarova. B. Petrovska IEB contributed to experiments design and to manuscript writing.
Petrovská, B., Cenklová, V., Pochylová, Z, ***Kourová, H., Doskočilová A., Plíhal, O., Binarová L., and Binarová, P***. (2012).Plant Aurora kinases play a role in maintenance of primary meristems and control of endoreduplication. New Phytologist 193, 3, pp.590-604, 2012 IF 6.736

Our protein interaction studies of TPX2, alfa-importin, Ran GTPase and gamma-tubulin together with colocalization analyses showed that TPX2-mediated microtubule formation might be triggered by a Ran cycle. **We described for the first time gamma-tubulin and TPX function in chromatin mediated nucleation of intranuclear microtubules in *Arabidopsis* and suggested multiple roles for TPX2 with gamma-tubulin in acentrosomal microtubule organization.** A part of experimental work was performed in IEB, graduate students supervised by P. Binarova (Gelova, Vachova) contributed substantially to microscopy and apoptosis labelling, B. Petrovska contributed to manuscript writing.

Petrovská,B. Jeřábková, H., *Kohoutová, L.,*Cenklová, V., Pochylová, Z., Gelová, Z., *Kočárová, G.,* Váchová, L., Kurejová, M., Tomaštíková, E., ***Binarová. P. (***2013) Overexpressed TPX2 causes ectopic formation of microtubular arrays in the nuclei of acentrosomal plant cells. J. of Exp. Botany, Vol. 64, No. 14, 4575–4587. IF 5.794

(2) We performed extensive proteomic analyses of gamma-tubulin interactom in acentrosomal *Arabidopsis* cells and we studied novel interactors and their cellular functions

(a) We characterized molecular forms, localization, protein interactions and function of Nodulin/glutamine synthetase-like protein (NodGS) which was identified in our proteomics studies of gamma-tubulin interactors as yet uncharacterized plant homologue of fungal FluG protein. Using RNAi, biochemical analyses, GFP approach, Y2H screening and biotic stress signalling including M|AP kinase assays, we found a dual role for NodGS in root morphogenesis and microbial elicitation. P.Halada (IM) performed MS analyses, B.Petrovska (IEB) performed QPCR. **Our data showed that the family of NodGS/FluG-like fusion genes is widespread in prokaryotes, fungi, and plants and suggested their cellular function.**

Doskočilová, A., Plíhal O., Volc J., Chumová, J., Kourová, H., Halada P., Petrovská, B., ***Binarová P.*** (2011) A nodulin-glutamine synthase-like fusion protein is implicated in the regulation of root morphogenesis and in signalling triggered by flagellin. Planta 234: 459-476. IF 3.0

(b) We found that as yet uncharacterized *Arabidopsis* homologue of RanBPM protein, previously reported as a player in chromatin mediated nucleation of microtubules, physically interacts with LisH-CTLH domain-containing proteins. Using biochemical analyses and protein interaction studies and CLSM analyses we showed **that newly identified protein complexes of AtRanBPM are homologous with CTLH complexes known from other eukaryotes and the conservation suggested important biological role of the complexes**. The first author (shared first authorship) and corresponding author are from our team, P. Halada (MBU) performed MS analyses.Database search, cloning, microscopy were performed in IEB where E. Tomaštikova contributed substantially to MS writing.

Tomaštíková, E., Cenklová, V., Petrovská, B., ***Kohoutová, L.,*** Váchová, L., Halada, P., ***Kočárová, G., Binarová, P.*** (2012). Interactions of an *Arabidopsis* RanBPM homologue with LisH-CTLH domain proteins revealed high conservation of CTLH complexes in eukaryotes. BMC Plant Biol, 12:83. IF 4.354

(3) Using biochemistry, RNAi and GFP approaches, flow cytometry, expression analyses and microscopy we showed that Arabidopsis nitrilase1 NIT1 is essential to maintain ploidy level, genome stability and tissue organisation. **Our data on sustained proliferation, and polyploidy in cells with depleted Nit1and in opposite increased rate of apoptosis in cells over-expressing Nit1 suggested that Nit1/Fhit oncogene pathway of animals is conserved in eukaryotes**. MS analyses were performed by P. Halada, EM by O.Benada (both MBU), QPCR analyses by B. Petrovska (IEB). L.Bogre (UK) contributed to design of experiment and writing.

Doskočilová, A., Kohoutová L., Volc, J., Kourová, H., Benada O., ***Chumová, J., Plíhal O.,*** Petrovská, B., Halada P., Bogre L., ***Binarova P.*** (2013) Nitrilase 1 regulates the exit from proliferation, genome stability and plant development. New Phytologist, 198 (3) 685-698. IF 6.736.

(4) We found that active mitogen-activated protein MAP kinases colocalized with gamma-tubulin on the specific subset of mitotic microtubules and MPK6 kinase interacted with gamma-tubulin in Arabidopsis cells. Microtubule plus-end binding protein EB1was associated with and was phosphorylated by activated MPK6 *in vitro*, phosphorylation was confirmed *in vivo.* Cells with reduced MAP kinases activity due to overexpression of MAP kinase phosphatase AP2C3 showed impaired congression and separation of chromosomes. **Identification of novel protein targets of**

MAP kinase signaling among microtubular proteins will help to get insight into role of MAP kinases in modulation of cell division through cytoskeleton and in regulation of mitosis. Experimental work, design of experiments and writing of manuscript was performed by our team, P.Halada provided MS data, S Nagy. and T. Mészáros performed in vitro translation and phosphorylation, I Meskiene provided AP2C3 mutants and L Bogre contributed to manuscript writing.

Kohoutová L., Kourová H., Nagy S.K., ***Volc J.,*** Halada P., Mészáros T., Meskiene I., Bögre L., ***Binarová P***. The Arabidopsis mitogen-activated protein kinase 6 is associated with γ-tubulin on microtubules, phosphorylates EB1c and maintains spindle orientation under nitrosative stress. New Phytologist submitted 2014, revision submitted April 2015.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Cellular and Molecular Immunology

The team includes junior members, such as master and doctoral students, and early to middle career scientists, however we benefit from the experience of senior scientists (H. Tlaskalová, L. Tučková, J. Mestecky – all of them working part-time) who are involved in the training of more junior members. Our laboratory is focused on the cellular and molecular immunology from point of view of the basic research with a substantial clinical impact. In principle, the research is focused on all aspects of the interaction of microbiota with mucosal surfaces. The pertinence of such broad approach has resulted in the invitation to contribute by several chapters to the 4th edition of the book Mucosal Immunology (Mestecky et al., Eds, Elsevier, 2015).

Immunomodulatory and anti-inflammatory effect of microbial components

Gut microbiota plays significant role, both beneficial and deleterious, in the development of many inflammatory diseases (Tlaskalova-Hogenova H. et al. Cell Mol Immunol. 2011;8:110-20.; Kverka M. and Tlaskalova-Hogenova H., APMIS, 2013;121:403-421). This is best established in the inflammatory bowel diseases (IBD), where numerous interactions between host and microbe changes the susceptibility to the disease. Using experimental model of murine colitis, we showed that live probiotic bacteria can prevent severe forms of intestinal inflammation (Kokešová A et al., Folia Microbiol (Praha). 2006;51: 478-84). To use live microbes to treat inflammatory diseases has two major hurdles. First, any living microbe may be dangerous to the immunocompromised host and, second, storage and transport of viable microbes is more complicated than transport of lifeless drugs. Therefore, we focused on the immunomodulatory properties of lifeless lysates of well-known probiotic *Lactobacillus casei* DN-114 001 (Lc) and commensal anaerobic bacterium, *Parabacteroides distasonis* (Pd). First, we cultivated these lysates with the LPS-activated macrophages RAW 264.7 *in vitro*, where these lysates decreased their TNF-α production and NF-κB activity. To analyze the potential anti-inflammatory effect of these lysates, we fed immunocompetent BALB/c mice with four weekly doses of either Lc or Pd and induced intestinal inflammation with 3% dextran sulfate sodium (DSS) one week later. We found that oral treatment of mice with whole lysates of these microbes significantly reduce the severity of acute DSS colitis. We did not observe this protective effect, when mice were fed with lysates of other microbes (e.g. *L. plantarum*, *B. thetaiotamicron*, *V. alcalescens*, *B. ovatus*, *B. vulgatus* and *B. stercoris*) in a similar way. The treated mice had significant changes in their gut microbiota composition and prevented DSS-induced increases in several pro-inflammatory cytokines in gut mucosa and increased proportion of regulatory T cells in mesenteric lymph nodes. In these lysates, this effect was dependent on presence of adaptive immunity mechanisms, because it disappeared in immuno-deficient SCID mice. There were some differences between both lysates, because oral feeding with Pd (and its membraneous fraction (mPd) induced systemic antibody response and the protective effect could be transferred with serum. Our study shows that specific bacterial components derived from the probiotic or commensal bacteria have strong anti-inflammatory and immunomodulatory mechanisms *in vitro* and *in vivo*. These sterile microbial components may be source of the new IBD therapeutics, which may be safer as

compared to live bacteria (Kverka M. et al. Clin Exp Immunol. 2011;163:250-9.; Zákostelská et al. PLoS One, 2011, 6: e27961). These studies were performed in our laboratory in collaboration with Laboratory of Gnotobiology, where experiments with immunodefficient animals were performed, and with Institute of Animal Physiology and Genetic (IAPG) and Institute of Molecular Genetics (IMG), where microbiota was analyzed.

Gut microbiota and carcinogenesis

Gut microbiota plays significant role in the transition of chronic intestinal inflammation to neoplasia (Tlaskalova-Hogenova H., et al. Cancer J., 2014, 20:217-24). To investigate the impact of bacterial load on carcinogenesis, we induced colitis-associated cancer by azoxymethane/dextran sodium sulfate in conventional, germfree and antibiotic-treated mice, and investigated the underlying mechanisms. We found that germfree and antibiotic-treated wild-type mice had lower incidence and severity of tumors. We collected stool samples of mice with and without antibiotic treatment in the day of azoxymethane injection and analyzed the microbiota composition by 454 pyrosequencing and by quantitative RT-PCR, and activity of beta-glucuronidase by fluorometric assay. We found that ATB treatment significantly changed the gut microbiota composition and markedly decreased the beta-glucuronidase activity. Both these results are in agreement with each other since there is decrease in bacteria known to produce beta-glucuronidase in ATB-treated mice (e.g. Clostridiaceae). We conclude that certain microbes promote colon carcinogenesis by releasing the azoxymethane, carcinogen broadly used for the induction of experimental colorectal cancer, and possibly other naturally occuring carcinogens from their innocent glucuronide conjugate by beta-glucuronidase. Therefore the bacteria harboring beta-glucuronidase can promote the production of carcinogen in the gut with further severe tumor development. In conclusion, antibiotic treatment reduced tumor development by decreasing the load of gut bacteria with beta-glucuronidase activity. Since negative regulation of Toll-like receptor (TLR) signaling is known to influence the development of colorectal cancer, we induced colitis-associated cancer in mice lacking key negative regulator of TLR signaling, Interleukin-1 receptor-associated kinase-M (IRAK-M). IRAK-M-/- mice not only developed invasive tumors, but alteration of gut microbiota by antibiotics did not rescue them from severe carcinogenesis phenotype. Moreover, tumorigenesis promoted chronic inflammatory status in these mice as analyzed by serum haptoglobin and production of pro-inflammatory cytokines in the colon. We conclude that IRAK-M molecule is important for regulation of excessive inflammatory response and for effective anti-tumor immunity (Klimešová K. et. al. Inflamm Bowel Dis. 2013;19:1266-77). Most of these experiments were performed in our laboratories; we collaborated with Laboratory of gnotobiology on gnotobiotic experiments and with IAPG and IMG on microbiota analysis.

Next, we analyzed, which pathways are involved in the colorectal carcinogenesis. We found that Troy, negative modulator of the Wnt pathway, is significantly upregulated in fast-cycling intestinal stem cells, in mouse tumor tissue and in samples of human colorectal cancer (Fafilek B et al. Gastroenterology. 2013;144:381-91). These findings are result of our long lasting collaboration with dr. Korinek's group from IMG. Our laboratory participated by experiments in mouse model of colorectal cancer

Maternal immune factors influence the development of newborns: antibodies present in colostrum react with broad spectrum of antigens

Analysis of perinatal influences of human development, and breastfeeding in particular, belong to one of our research interests (Vancikova Z, Folia Microbiol (Praha). 2003;48:281-7.; Kverka M. et. al. Clin Chem. 2007;53:955-62.; Zizka J, et al. Folia Microbiol (Praha). 2007;52:549-55.). By providing both nutrients and numerous immune components, human lacteal secretions (colostrum and milk) aid the newborn to adapt to the environment. These factors provide the newborn's immature immune system with means to resist many infection risks encountered after birth. Among many humoral immune factors present in human lacteal secretions, secretory IgA (SIgA) plays an important role. Secretory IgA antibodies in colostrum represent the first line of defence against harmful substances, but their potential spectra of reactivity with autoantigens remains unclear. We characterised the repertoire of natural sectretory IgA antibodies in colostrum of healthy mothers. The human colostrum samples from healthy mothers were analyzed for antibodies by indirect immunofluorescence, dot blots, immunoblots and ELISA.We found that there is high diversity in reactivities of colostral IgA antibodies: beside bacterial components various autoantigens as target antigens were demonstrated. Using tissue sections and biochips commonly used for autoimmunity testing, we found that most samples reacted with ovary, pancreatic tissue, HEp-2 line cells and adrenal gland, fewer samples reacted with liver tissue, stomach, testicular tissue, monkey salivary gland, rat kidney and cerebellar tissue. Using immunoblotting and ELISA we detected reactivity of IgA with 21 isolated, defined antigens. The majority of the samples reacted with the pyruvate dehydrogenase complex, E3 ubiquitin ligase, cytosolic liver antigen, and nuclear pore glycoprotein-210, nuclear antigens, double stranded DNA, phospholipids and neutrophil cytoplasm. The broad spectrum of polyreactive natural antibodies present in human colostrum may contribute to proper development of mucosal immune system of the breastfed infant (Přibylová J. et. al. J Clin Immunol. 2012 Dec;32(6):1372-80). These experiments were performed in our laboratory and we obtained samples of human milk and colostrum from Institute for the Care of Mother and Child.

Celiac disease pathogenesis

The overgrowth of potentially pathogenic bacteria and infections are suggested to contribute to celiac disease pathogenesis. In cooperation with colleagues from Valencia, we demonstrated, using our gnotobiotic model, that gluten fragments, IFN-γ and intestinal bacteria enlarged in patients, can reduce the number of goblet cells, mucin secretion, epithelial integrity and thus increase translocation of inducing agent, gluten into lamina propria. On contrary, some *Bifidobacteria* enhance production of chemotactic factors and inhibitors of metalloproteinases, and contribute to mucosal protection. (Cinova J. *et al. PloS One 6(1): e16169, 2011).* Furthermore, we have shown that *Enterobacteria* and/or gliadin induce alteration in dendritic cell (MDDC) morphology, migratory phenotypes and increase inflammatory cytokine production, in contrast to *Bifidobacteria* , which increase regulatory cytokine IL-10 secretion and tend to reverse gliadin-induced IFN-□ production and epithelial tight junctions reduced expression (De Palma G.et al. *J. Leukoc. Biol.* 92: 1043-1054, 2012).

In close cooperation with Medical University and Max Planck Institute in Freiburg we have demonstrated that blood mononuclear cells and monocytes from celiac patients respond to pepsin digested wheat gliadin fraction (PDWGF) by robust secretion of IL-1b and IL-1a and low production of IL-18. The role of inflammasome receptor NLRP3 and apoptosis-associated ASC, was supported by dendritic cells isolated from knockout mice. Moreover, we

suggested involvement of TLR2/4/MyD88/MAPK signaling molecules in cell stimulation (Palová-Jelínková L. *et al*. *Plos One* 8(4): e62426, 2013).

Copolymer P(HEMA-co-SS) was developed to reduce digestion of wheat gluten and barley hordein, and thereby formation of toxic peptides and immune response associated with celiac disease. We were invited to cooperate on this topic by J-Ch. Leroux (Zurich). We found that after oral administration in rodent copolymer was excreted in feces even in low-grade mucosal inflammation. Furthermore, that secretion of inflammatory cytokine TNF-□□ by mucosal biopsy specimens from celiac patients induced by digested gliadin could be reduced in the presence of copolymer (Pinier M.*et al. Gastroenterology* 42:316-325.e12 , 2012).

Gliadins, are critical in the induction of the damaging immune response that leads to the development of celiac disease. In cooperation with CNRS Limoges (M. Cogné) and Uni-Liepzig (T. Mothes) We prepared several humanized monoclonal IgA anti-gliadin antibodies using transgenic α1KI mice and, by employing Pepscan with peptides of α-gliadin, we observed an important similarity between the fine specificity of humanized mouse monoclonal IgA anti-gliadin antibodies and IgA anti-gliadin antibodies from patients with florid celiac disease. The antibody similarity enables us to use these humanized antibodies for both diagnostic and experimental purposes (Sánchez D. *et al*. *J Agric Food Chem*. 59:3092-100,2011).

We described for the first time the occurrence of IgA and IgG antibodies against calreticulin (CRT) in sera of some patients with gastrointestinal malignancies – mainly with hepatocellular carcinoma, colorectal adenocarcinoma and pancreatic adenocarcinoma. Simultaneously, we performed analysis of B-cell epitopes using Pepscan technique and revealed a number of antigenic epitopes of CRT recognized by both IgA and IgG antibodies of patients differing in diagnosis (Pekáriková A et al., *Clin Exp Immunol.* 160:215-222,2010).

Translocation of CRT on the surface of dying tumor cells is considered crucial for induction of antitumor immune response, which could include also induction of anti-CRT antibodies. The role of anti-calreticulin antibodies is unknown; however, these antibodies could negatively influence the tumor cell recognition and engulfment by immune cells. We were asked by L. Zitvogel (France) to analyze the presence of anti-CRT antibodies in sera of patients with neoadjuvantly treated breast carcinoma. Our results documented no influence of neoadjuvant treatment on induction of anti-CRT Ab in these breast carcinoma patients Hannani D et al., *Cell Death Differ*. 21:50-58, 2014).

Food allergy

Food allergy affect 6-8% of young and 2-4% of adults. The specificity and sensitivity of diagnostic tests depends on purity of allergens. We have developed new technique (preparative isoelectric focusing and liquid chromatography) to isolate wheat allergens in their native form. From 27 potential wheat allergens, identified by proteomic approach, 7 were newly reported and were included into the list of allergens ISMA-Allergome. The frequency of IgE reactivity of patients with isolated molecules led to selection of 4 for further clinical testing (Šotkovský P. et al. Clin. Exp. Allergy 41:1031-43, 2011). *Rice diet is often recommended in food allergies. We analysed IgE binding rice water-salt soluble and insoluble proteins of patients with food and pollen allergies. Using mass spectrometry we have identified 22 rice SDS soluble IgE binding molecules, 6 of them was thermostabile potential rice allergens (2 added to ISMA-Allergome). We suggested in patients with the highest IgE in ELISA and immunoblotting and positive basophil activation to perform the skin prick test with boiled rice proteins before diet recommendation (Goliáš et al. J. Agric. Food*

Chem. 61:8851-8860, 2013). Allergies to eggs, milk and peanuts are currently most frequent and their prevalence, severity and persistence has been increasing in the last decades. In mouse model of allergy to egg ovalbumin (OVA) we demonstrated the impact of thermal processing on allergy development. The increased temperature (to 70^0C or 95^0C) caused irreversible changes in OVA secondary structure, kinetics of digestion and antigenic peptides formation, leading to activation of different T cell subpopulations in in vitro studies. Balb/c mice were sensitized intraperitoneally and challenged with repeated gavages of OVA heated 70^0C (h-OVA) or native OVA. Heating of OVA significantly decreased clinical symptoms and immune response on the level of IgE, IL-4, IL-5 and IL-13, mast cell protease-1 and eneterocyte brush border membrane alkaline phosphatase, but stimulated higher IgG2a in sera and IFN–γ secretion by splenocytes, demonstrating the shift towards Th1 immune response (Goliáš J. et al. *Plos One* 7(5), e37156, 2012).

To investigate the effect of intestinal bacteria on food allergy development OVA model was transferred to germ-free conditions (Laboratory of Gnotobiology) and the impact of monocolonization with probiotic bacteria and conventional condition (ex-germ-free) on immune response and the onset of allergy symptoms and currently studied.

Type 1 diabetes

In the period of last 5 years (2010-2014) our group has dealt with above all projects focused on the role of environmental factors such as diets and microbioms in the pathogenesis and prevention of type 1 diabetes (T1D). We have investigated mechanism of prevention of T1D in NOD mice by gluten-free diet and described changes in mucosal T cell subsets and their cytokine signatures (Antvorskov et al.: PLoS One. 7(3):e333151, 2012; Antvorskov et al.: Immunology, 138;23-33, 2013; Antvorskov et al.: Diabetologia., 57: 1770-80, 2014) and tested the time-window (prenatal, weaning period etc.) that is important and sufficient for the diabetes prevention by gluten-free diet. In germ-free gnotobiological model of NOD mice we have initiated studies focused on the interplay of the mouse microbiom and diabetes-protective diets. Finally, we have tested intranasal administration of gliadin as a preventive and early–cure intervention for diminishing T1D incidence in NOD mice (Funda et al.: PLoS One. 9(4):e945304, 2014). Prevention of T1D by intranasal gliadin, an environmental antigen with possible etiological role in T1D, may represent a novel, safer strategy for prevention or even early cure of T1D.

Early evolution of mucosal immunity

The origin and development of mucosal surfaces, particularly the absorptive tissue associated with the intestine, represent the major evolutionary step that supported multicellular life and allowed the development of body pattern, organs and organ systems. In our laboratory, we focus on the study of innate immunity in annelids. The earthworms host a number of discrete bacterial communities depending on the ecological niches that colonize. The number of bacteria varies from approximately 10^6 CFU per gram dry weight of the substrate in soil to more than 10^8 CFU per gram dry weight in compost. It is estimated that a single earthworm swallows daily approximately the same mass of soil substrate equal to its weight, i.e., that 10^8 bacteria pass through the gut interacting with and affecting the mucosal surface of the gut. From this point of view, earthworms provide a useful model to study evolutionarily ancient mechanisms of the innate immunity in the gut (Procházková et al., J. Invertebr. Pathol. 114, 217-221, 2013; Dvořák et al., PLoS ONE 8, e79257, 2013).

We have described the differential expression of pattern recognition receptors (CCF, LBP, TLRs) in the gut and the correlation between their expression with enzyme activities in the gut of *Eisenia andrei* earthworms following a microbial challenge (Škanta et al., *Dev. Comp. Immunol.* 41, 694-702, 2013; Procházková et al., J. Invertebr. Pathol. 114, 217-221, 2013; Procházková et al., PLoS ONE 9, e10, 2014; for review Bilej et al., Invertebrate Immunology, 2010). The data suggest that enzyme activities important for the release and recognition of molecular patterns by pattern-recognition molecules, as well as enzymes involved in effector pathways, are modulated during the microbial challenge. Finally, we have suggest the antimicrobial immune response in the gut and its implication to the sensitivity to soil organopolutants (Roubalová et al., *Environ. Pollut.*. 193, 22-28, 2014).

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Tumor Immunology

Development of novel class of cancer therapeutics based on *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers of various structures as a carriers of anticancer drugs, predominantly doxorubicin, is a long-term goal of our laboratory. Polymeric carrier could be either simple linear HPMA copolymer with Mw ~ 30.000 kDa or much bigger molecules like star structure where the PAMAM dendrimeric core is substituted with multiple linear HPMA copolymers making a structure of Mw ~ 200.000 kDa. Doxorubicin or other drugs (for example paclitaxel) could be bound to the polymeric carrier either through proteolytically cleavable amide bond (Dox-HPMA^{AM}) or through pH-sensitive hydrazone bond (Dox-HPMA^{HYD}) which dramatically affects resulting biological properties and anticancer activity of such conjugates. It seems that conjugates with pH-dependent hydrolytically cleavable bond between the drug and carrier are more promising. These polymer-bound drugs exert significantly improved pharmacological properties in comparison to low-molecular weight drugs including their passive accumulation in solid tumors due to the enhanced permeability and retention effect. Moreover, these conjugates could be equipped with targeting moiety like mAb or specific synthetic peptide in order to increase the specificity of cytotoxic effect toward tumor cells. We have demonstrated in two syngeneic mouse tumor models that these conjugates are able to completely cure mice with progressively growing tumors and that these mice establish long-lasting resistance specific to original tumor. The synthesis and characterization of these conjugates is carried out by Department of Biomedical Polymers (Prof. Karel Ulbrich DSc.) at Institute of Macromolecular Chemistry (IMC) while testing both *in vitro* and *in vivo* is carried out in our laboratory.

Second general aim of our laboratory is the study of biological activities and mechanism of action of IL-2 immunocomplexes formed by anti-mouse IL-2 mAb (S4B6, JES6-1) and mouse IL-2 when co-incubated at molar ratio 1:2. These IL-2 immunocomplexes possess very high biological activity *in vivo* if compared to IL-2 alone. Furthermore, depending on the clone of anti-IL-2 mAb used, these IL-2 immunocomplexes exert selective stimulatory activity toward different IL-2 responsive cell subsets. IL-2/S4B6 mAb immunocomplexes are highly stimulatory for CD122^{high} populations (memory CD8^{+} T and NK cells) and intermediately also for CD25^{high} populations (T_{reg} and activated T cells) while IL-2/JES6-1 mAb immunocomplexes enormously expand selectively CD25^{high} cells. IL-2 immunocomplexes are thus useful and potent tool for modulation of immune system and immune responses and could be also successfully used in treatment of experimental mouse tumors in combination with HPMA copolymer-bound drug conjugates.

We published the review regarding the current knowledge about HPMA copolymers as carriers of cytostatic drugs with respect to their physico-chemical properties, safety/toxicity and antitumor activity. We also discussed their quite unique feature that during the treatment with HPMA copolymer bound doxorubicin conjugates the induction of potent and long-lasting tumor-specific immunity is commonly seen (Rihova B., Kovar M., Adv Drug Deliv Rev. 2010, Feb 17; 62(2):184-91).

We showed for the first time that two slightly different conjugates containing doxorubicin covalently bound either through amide bond or through hydrazone bond to HPMA copolymer carrier synergize in their antitumor activity *in vivo*. Synthesis and characterization of these conjugates was carried out at IMC. Our laboratory was responsible for planning and performing all biology relevant experiments and methodology. We also analyzed all biology data and wrote manuscript (Ríhová B. et al., Mol Pharm. 2010, Aug 2; 7(4):1027-40). Further, we summarized our preclinical evaluation of linear HPMA-based conjugate carrying doxorubicin bound via the hydrazone bond. Parameters such as drug payload, critical content of free drug and molecular weight related to therapeutic efficacy were shown in murine tumor model together with treatment-induced antitumor immunity (Sirova M. et al., Pharm Res. 2010, Jan; 27(1):200-8). Our laboratory was responsible for all work except synthesis and characterization of polymeric conjugates (IMC).

Unexpectedly, we identified very crucial flaw in fluorescence-based intracellular detection methodology of doxorubicin caused by its derivate D. We also proposed alternative microscopy and flow cytometry methods for its accurate detection. It allows to correctly asses doxorubicin fluorescence intensity and not the incorrect Dox and derivate D fluorescence intensities sum (Hovorka O. et al., Eur J Pharm Biopharm. 2010, Nov; 76(3):514-24). Our laboratory did all experiment leading to the creation of the alternative methods, together with preparation of the manuscript.

Our group also cooperated with other European polymeric chemists on development of implantable polyimide sheets (PolExGene project) intended for regenerative medicine application (Van Vlierberghe S. et al., Biomacromolecules. 2010, Oct 11; 11(10):2731-9). This paper was a basis for subsequent work of a consortium of several laboratories within the (see S. Julien et al, Biomaterials 2011, Jun; 32:3890-3898). We performed the studies on bio- and immunocompatibility of the sheets including the intermediate steps in the development to ensure the final applicability of the devices *in vivo*.

Next, we studied the effect of IL-2/S4B6 immunocomplexes on various immune cell population and we demonstrated that anti-tumor activity of these immunocomplexes synergize with that of HPMA copolymer-bound doxorubicin conjugates (Tomala J. et al., Int J Cancer. 2011, Oct 15; 129(8):2002-12). Only few other manuscripts cover the topic of anti-tumor activity of IL-2 immunocomplexes. The study shows important findings for the field. Our group did all the experiments except of HPMA copolymer-bound doxorubicin conjugates synthesis and characterization, and we also prepared the manuscript.

In another study, we have designed, produced and tested new biodegradable HPMA copolymer-based star conjugates containing doxorubicin with PAMAM core designed for passive tumor targeting (Etrych T. et al., J Control Release. 2011, Sep 25; 154(3):241-8). This manuscript brings new findings to the design of HPMA copolymer-bound drug conjugates and leads to synthesis of conjugates fully eliminable from the organism. Our lab members performed biological testing *in vitro* and *in vivo* including tumor-survival experiments in experimental mouse models. Simultaneously with this work, we studied the effect of molecular weight, polydispersity, hydrodynamic radius, and molecular architecture of various HPMA copolymer conjugates on the biodistribution and treatment efficacy *in vivo*. The finding that linear polymer conjugates with molecular weight near 70 kDa could be excreted from the body via renal elimination, even though the generally accepted limit for kidney filtration is 45 kDa is highly interesting and novel (Etrych T. et al., J Control Release. 2012, Dec 28; 164(3):346-54). Our laboratory did all biology relevant work.

To investigate and improve the biological features of IL-2/S4B6 mAb complexes, we designed and produced protein chimera consisting of IL-2 linked to light chain of anti-IL-2

mAb S4B6 through flexible oligopeptide spacer (Gly4Ser)3 and performed biological and immunological testing of this compound. The importance of this manuscript lies in the finding that IL-2/S4B6 protein chimera possesses comparable or even higher biological activity with more favorable conditions for IL-2 control and behavior *in vivo*. There are no coauthors out of our lab.

In our other work, we have delineated one of the potential mechanisms of anti-tumor immune response induced via treatment with HPMA-copolymer conjugates, i.e. induction of immunogenic cancer cell death (Sirova M. et al., Curr Med Chem. 2013, 20(38):4815-26). To date, it is our most comprehensive study of the immune mechanisms that are essential for the complete cure of cancer, using treatment with HPMA-based conjugates. Predominant part of the work was done in our laboratory, as conjugates described earlier and now routinely synthesized were used in the study. Last but not least, we have confirmed that water-soluble HPMA copolymers conjugates could be used to simultaneously deliver a cytostatic drug and a P-gp inhibitor to tumor and thus these conjugates represents promising strategy to overcome multidrug resistance in cancer cells. Here we introduced for the first time conjugate containing both cytostatic drug and P-gp inhibitor in one molecule (Subr V. et al., Biomacromolecules. 2014, Aug 11; 15(8):3030-43). Our laboratory has participated in overall design of the study, all biology relevant experiments and methodology and writing the manuscript.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Gnotobiology

Part of the laboratory under leadership of **Marek Sinkora** has primarily concentrated to study the B and γδ T lymphocyte development in swine. The studies of B cell development were initiated by a finding that the process of B cell lymphogenesis in swine remained uncertain. Pigs belonged historically to a group of animal that uses ileal Peyer's patches (IPP) for antigen-independent generation of B cells. However, our previous data were in conflict with this presumption. In cooperation with the University of Iowa and South Dakota State University we have designed series of experiments to demonstrate dispensability of IPP for B cell development (Butler et al. 2011). The results disprove the concept that porcine IPP are a significant source of B cells, are required for maintenance of the systemic B cell pool and/or are a site of B cell lymphogenesis in swine (Sinkora et al. 2011). Following of initial finding, our laboratory independently of foreign cooperation has also clearly shown that maturation of B cells in IPP is antigen-dependent (Potockova et al. 2015). These findings disprove the paradigm about the role of IPP in ungulates that was maintained for over 30 years in other scientific reports, reviews, and immunology textbooks. We have summarized all findings and arguments in recent review (Butler and Sinkora 2013). On the other hand, using flow cytometry sorting and semi-quantitative PCR detection of rearrangement-specific transcripts and DNA products we show that a major B lymphopoietic organ of pigs is the bone marrow (Sinkora and Sinkorova 2014). As a side effect of studies we have also shown that λ rearrangement precedes κ rearrangement during B-cell lymphogenesis in swine (Sun et al. 2012). These recent works resolved the course and differentiation pathways of B cell maturation in the bone marrow.

Our studies of B lymphocytes in pigs also involve characterization of B cell maturation in the periphery because there is no study that would discriminate functionally different subpopulations of porcine B cells. Our ontogenetic and in vitro culture studies, analysis of cell size, expression of CD11b and class-switched phenotype together with measurement of proliferation and cell death, revealed four subpopulations of functionally different B cells in the periphery according to expression of CD2 and CD21 (Sinkora et al. 2013). Moreover, we have shown that CD21 molecules are always present on all mature B cells but can be expressed in at least two differential forms CD21a and CD21b. We have also shown that end-stage B cells can express differential form of CD21, which can be significant for their function (Sinkora et al. 2013).

Our investigation of porcine γδ T lymphocytes revealed that expressions of CD25, CD11b, CD52, SWC7, MHC-II and family of CD45 molecules on these cells differ in dependence on bacterial colonization, age of animals and status of cell activation (Stepanova and Sinkora 2012). These studies implied that CD2/CD8 subsets of porcine γδ T cells may represent functionally different cells. For this reason, these subpopulations of γδ T lymphocytes were studied in more detail. Sorting and

following in vitro cultivation showed that CD2 expression can be used for definition of two lineages of γδ T cells (Stepanova and Sinkora 2013). Because CD2^{-} γδ cells are missing in the blood of humans and mice, but are obvious in other members of γδ-high species such as ruminants and birds, our findings support the idea that circulating CD2^{-} γδ T cells are a specific lineage.

A part of work that concern study of γδ T cells involved investigation of murine γδ T cells expressing canonical Vδ1-Dδ2-Jδ2 chain which completely lacks junctional diversity (Holtmeier et al. 2010). We showed that γδ T cells expressing this canonical TCRδ chain are not unique to the skin and reproductive sites. We also discover other γδ T cells expressing fetal type of Vδ1 chains, which were shared among different organs and animals. These findings indicate that γδ T cells expressing conserved Vδ1 chains continuously recirculate throughout the organism and rapidly respond to stress-induced self antigens.

In cooperation with the University of Iowa Carver College of Medicine we also participated on investigation of inflammatory cells that participate in destruction of the pancreas during course of cystic fibrosis (CF). In CF, pancreatic disease begins *in utero* and progresses over time to complete destruction of the organ. This is unusual type of autoimmune inflammation that develops in fetal live. Study was performed on CFTR gene knock-out pig model because CFTR$^{-/-}$ mice do not develop pathological changes similar to humans. We discovered the activation of both the innate and adaptive immune systems during destruction of pancreas indicating that immune reaction may be initiated during fetal life, in a period of time when only tolerance should be expected (Abu-El-Haija et al. 2011).

Last element of research was concentrated to study of three leading causes of disease in pigs worldwide: swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV) or porcine circovirus type 2 (PCV2). These viruses are responsible for significant economic losses with an estimated annual loss to PRRSV alone approaching 1 billion dollars just in the USA. We have chosen unique approach of germ-free animal model in which only the virus can be responsible for the immune changes. For the first time we reported on the phenotypic profile of T, B and NK cells and their subsets utilizing our recent knowledge about these lymphoid cells in swine allowing for comparative analysis of over 20 different lymphoid subpopulations (Sinkora et al. 2014). The results suggest that PRRSV and PCV2 may negatively modulate the host immune system by different mechanisms, which was summarized in a separate report (Butler et al. 2014).

Part of the laboratory under leadership of **Renata Stepankova** has concentrated to study the effect of bacterial colonization and diet on the development of atherosclerosis in ApoE-deficient C57BL/6 mice. Atherosclerosis - a chronic inflammatory disease of large and medium arteries is characterized by lipid deposition in arterial walls. Apolipoprotein E-deficient (ApoE deficient) mice, a widely used mouse model of human atherosclerosis, have hyperlipidemia and develop all phases of atherosclerotic lesions which are seen in humans. Our experiments were performed on ApoE-deficient mouse strain C57BL/6, bred under germ-free (GF) conditions for two generations or under conventional (CV) conditions with defined microflora. Mice were fed a standard low cholesterol diet or cholesterol-rich. We studied the development of advanced lesions in the thoracic and abdominal aorta by histological, morphometric and immunohistological methods. ApoE deficient mice

reared under germ-free conditions for two generations and fed as high as low cholesterol diet exhibited atherosclerotic plaques in the aorta. Characteristic lipid deposition with foam cells and macrophages was found in their arterial walls. In contrast to the absence of atherosclerotic plaques in conventionally reared ApoE-deficient mice, germ-free ApoE-/- mice consuming the same low cholesterol standard diet, developed atherosclerotic plaques in the aorta. Our findings thus documented that absence of microbiota (germ-free conditions) accelerates the atherosclerosis in ApoE-deficient mice fed standard low cholesterol diet (Stepankova et al. 2010).

In our work we use gnotobiotic animals as the major tool for host-microbe interaction studies. The crucial role of microbiota in the initiation and progression of inflammation-associated colorectal neoplasia were documented in Tlaskalova-Hogenova et al. 2011 and Klimesova et al. 2013. Carcinogenesis in the gut is driven by the presence of potentially harmful microbes or by lack of protective ones, by the production of carcinogens generated by microbes, and by the induction of inflammation and modulation of the immune system. Colitis-associated cancer was induced by azoxymethane and dextran sodium sulfate in wild-type and in interleukin-1 receptor-associated kinase M (IRAK-M)-deficient mice with or without antibiotic (ATB) treatment. ATB treatment of wild-type mice reduced the incidence and severity of tumors. Compared with nontreated mice, ATB-treated mice had significantly lower numbers of regulatory T cells in colon, altered gut microbiota composition, and decreased β-glucuronidase activity. However, the β-glucuronidase activity was not as low as in germ-free mice. IRAK-M-deficient mice not only developed invasive tumors, but ATB-induced decrease in β-glucuronidase activity did not rescue them from severe carcinogenesis phenotype. Furthermore, IRAK-M-deficient mice had significantly increased levels of proinflammatory cytokines in the tumor tissue. We conclude that gut microbiota promotes tumorigenesis by increasing the exposure of gut epithelium to carcinogens and that IRAK-M-negative regulation is essential for colon cancer resistance even in conditions of altered microbiota. Therefore, gut microbiota and its metabolic activity could be potential targets for colitis-associated cancer therapy.

In the next study we focused on effects of bacteria (*Bifidobacterium* and G- bacteria strains) isolated from celiac patients on immune changes in small intestinal loops of germ-free rats. The Gram-negative bacteria induced higher secretion of Th1-type proinflammatory cytokines (IL-12 and/or IFN-gamma) than the *Bifidobacterium* strains. *Shigella* CBD8 and *E. coli* CBL2 up-regulated mainly HLA-DR and CD40 expression involved in Th1 activation, and *Bifidobacterium* strains up-regulated CD83 expression. Specific interactions among the studied bacteria, gliadins, and IFN-gamma, which favored the CD immune features, were also detected. Therefore, intestinal bacteria could be additional factors that regulate the ability of monocytes recruited to the mucosa to respond to gliadins and IFN-gamma in celiac disease patients, influencing the course of the disease (De Palma et al. 2010).

Part of the laboratory under leadership of **Tomas Hrncir** has collaborated with Dirk Haller (Nutrition and Immunology, Technische Universität München, Freising-Weihenstephan, Germany; ZIEL - Research Center for Nutrition and Food Sciences, Freising-Weihenstephan, Germany) who found that lactocepin, a protease produced by *Lactobacillus paracasei* (L. p.), selectively degrades pro-inflammatory chemokine IP-10 resulting in decreased intestinal inflammation. The lactocepin-expressing bacteria might be used in the treatment of IBD, but also other chronic inflammatory

diseases, including allergic eczema, psoriasis, and rheumatoid arthritis. Our group proved that L. p. does express prtP-encoded lactocepin in the cecum of L. p. monoassociated mice. Dr. Hrncir was responsible for breeding of germ-free and *Lactobacillus paracasei* prtP monoassociated mice, bacterial culture and gavage of *L. paracasei* strains, tissue collection for gene expression and western blot analysis, and immunophenotypization of immune cell populations using flow cytometry (von Schillde et al. 2012).

The regulatory mechanisms in the pathogenesis of inflammatory bowel diseases are still not well understood. We demonstrate that gut microbiota stimulate the colitogenic immune system through TLR and negative regulation of TLR signaling is essential in maintaining intestinal homeostasis. Our group showed in germ-free studies that the expression of negative regulator IRAK-M is driven by commensal microbiota. Dr. Hrncir was responsible for all the breeding and laboratory work carried out using germ-free and mono-associated Balb/c mice. Specifically, generation and breeding of germ-free Balb/c mice, *Lactobacillus plantarum* mono-association experiments, tissue collection for histological scoring, cell culture and gene expression analysis and flow cytometric analysis of mesenteric lymph node and colonic lamina propria cells (Biswas et al. 2011).

Part of the laboratory under leadership of **Tomas Hudcovic** has mainly concentrated to study of inflammatory bowel diseases, especially to deal with ulcerative colitis. Using gnotobiotic animal models of human diseases we demonstrated that impaired host regulation of mucosal responses to commensal bacteria plays an important role in the development of several inflammatory and autoimmune diseases (Tlaskalová-Hogenová et al. 2011).

The aim of the study was to investigate the effect of butyrate producing bacterium *Clostridium* (*C*.) *tyrobutyricum* on dextran sulfate sodium (DSS) induced colitis in mice. We have found that intrarectal administration of *C. tyrobutyricum* prevented appearance of clinical symptoms of DSS-colitis, restored normal MUC-2 production and did not change expression of TJ protein ZO-1. The production of IL-18 was dependent on immunocompetency of mice. *C. tyrobutyricum* treatment lead to decrease of TNF-α and IL-18 level in the descending colon of both SCID and BALB/c mice strains. Three-fold increase of n-butyric acid level and two-fold increase of propionic acid level were found in *C. tyrobutyricum*-DSS-treated SCID mice when compared with saline-DSS-treated mice. *C. tyrobutyricum* protection against destruction of mucosal barrier is equally effective in immunodeficient SCID mice and immunocompetent BALB/c mice. Manifestation of cytokines IL-18 and TNF-α in acute DSS-colitis depends largely on immune cell composition of the mouse host. (Hudcovic et al. 2012).

Correct function of intestinal barrier is essential for maintaining the homeostasis. The gut barrier function is significantly decreased by certain drugs, most notably non-steroide anti-inflammatory drugs, or by intestinal inflammation. This damage can be counteracted by oral treatment with non-living *Lactobacillus casei* and *Lactobacillus plantarum* which significantly strengthen the gut barrier function during acute DSS inflammation, suggesting that probiotic bacteria components may be important in protection against epithelial apoptosis or through preserving the mucus layer (Zakostelska et al. 2011).

In our experiments, we overcome these issues by colonizing germ-free mice with microbiota from biopsy taken from patients with active inflammatory bowel disease, and analyzed, if thus human-microbiota associated (HMA) mice develop intestinal inflammation spontaneously or after its induction with dextran sulphate sodium. We found, that HMA-associated mice do not develop spontaneous colitis and the induced colitis is very mild. Since the early germ-free postnatal period may significantly changes the immune response (Hansen et al. 2012), we then followed the HMA mice for several generations, analyzing the susceptibility to intestinal inflammation. We found that, although none of the mice developed spontaneous colitis, one line of mice became more susceptible to DSS-induced colitis at F4 generation. This change was associated with the appearance of the strong band belonging to *Clostridium difficile* and partial disappearance of the band belonging to the *C. symbiosum* on a denaturing gradient-gel electrophoresis. This is the first study that use mice colonized with human mucosa-associated microbiota and follow their susceptibility to colitis for several generations. Manuscript is under review in Plos One (Du Z., Hudcovic T., Mrazek J., Kozakova H., Srutkova H., Schwarzer M., Tlaskalova-Hogenova H., Kostovcik M., Kverka M.: Development of bacterial communities and intestinal inflammation in germ-free mice colonized with mucosa-associated bacteria from patients with ulcerative colitis, 2015).

In cooperation with Jerry Wells (Wageningen University, Netherlands) we studied immunomodulatory properties of *Faecalibacterium prausnitzii* strains in DSS model of acute colitis in mice and we have found that *Faecalibacterium prausnitzii* strain HTF-F and its extracellular polymeric matrix attenuate clinical parameters in DSS-induced colitis (Rossi et al. 2015).

Part of the laboratory under leadership of **Hana Kozakova** has primarily concentrated to study effect of probiotic bacteria on the development of allergies. Allergies have become a serious health burden in developed countries. The rapid increase of allergic diseases in humans is dependent on microbial deprivation early in life. Reduced bacterial diversity and lower counts of lactobacilli and bifidobacteria were found in gut of allergic children. This finding has been the rationale for administration of probiotic bacteria in prevention and/or therapy of allergy. Germ-free (GF) animals represent a unique tool to study the interaction of the host with one specific probiotic strain or with defined probiotic mixture and to investigate their impact on the development of the immune system. We showed that neonatal mono-colonization of GF mice with a *Lactobacillus plantarum* NCIMB 8826 producing the major birch pollen allergen Bet v 1 attenuates the development of birch pollen allergy later in life. The mechanisms involve a shift towards a non-allergic Th1 phenotype accompanied by increased regulatory responses. Monocolonization with the Bet v 1 producing *L. plantarum* induced a Th1 biased immune response at the cellular level, evident in IFN-γ production of splenocytes upon stimulation with Bet v 1. After sensitization with Bet v 1 these mice displayed suppressed IL-4 and IL-5 production in spleen cell cultures as well as decreased antibody responses (IgG1, IgG2a, IgE) in sera. This suppression was associated with up-regulation of regulatory markers (TGF-β, Foxp3, IL-10) at mRNA level in the spleen cells. Mono-colonization with recombinant *L. plantarum* specifically induced immunomodulation of allergic immune responses, supporting the concept of early immuno-imprinting using recombinant lactic acid bacteria (Schwarzer et al. 2011). In next study we investigated the effect of neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* ssp.

longum CCDM367 on subsequent allergic sensitization to Bet v 1. Our data demonstrated that perinatal mono-colonization with *B. longum* reduces allergic sensitization by activating regulatory responses, likely via TLR2 and MyD88 signaling pathways. Thus, *B. longum* might be a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans (Schwarzer et al. 2013).

We have selected three lactobacilli strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 out of twenty four strains isolated from the stool of healthy infants with resistance to gastric acids and bile salts and inhibitory activity against bacterial pathogens. The mixture of these strains (Lmix) showed synergistic effects in induction of anti-allergic Th1-type cytokines and regulatory cytokine TGF-β in human whole blood cell cultures compared to the levels induced by each single strain alone (Cukrowska et al. 2010). We have shown that *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, and *L. casei* LOCK0919 are recognized via TLR2 and NOD2 receptors and stimulate bone marrow-derived dendritic cells to cytokine production in a species- and strain-dependent manner. Colonization of GF mice with Lmix improved intestinal barrier by strengthening the apical junctional complexes of enterocytes and by restoring the structure of microfilaments extending into the terminal web. Mice colonized with Lmix and sensitized to Bet v 1 allergen showed significantly lower level of allergen-specific IgE, IgG1, IgG2a and elevated levels of total IgA in sera and intestinal lavages as well as increased levels of TGF-β compared to sensitized GF mice. Splenocytes and mesenteric lymph node cells of Lmix-colonized mice showed significant up-regulation of TGF-β after *in vitro* stimulation with Bet v 1. Thus the mixture of three lactobacilli strains shows a potential to be used in prevention of increased gut permeability and onset of allergies in humans (Kozakova et al. 2015).

In collaboration with group of Andrzej Gamian (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Department of Immunology of Infectious Diseases, Polish Academy of Sciences, Wroclaw) we studied effects of polysaccharides isolated from *Lactobacillus rhamnosus* LOCK 900 on cell immunomodulation. Our study showed that *L. rhamnosus* produces polysaccharides (L900 2, L900 3) which differ greatly in their structure and ability to modulate the bone marrow-derived dendritic cells immune response to well-known probiotic bacteria *L. plantarum*. We conclude that polysaccharides, probably due to their chemical features, are able modulate immune responses by regulatory IL-10 production (Gorska et al. 2014).

We investigated the effect of thermal processing on food allergy induction and on the main features of immune responses to native and heated form of ovalbumin (OVA) in mice. Heating led to slight irreversible changes in OVA secondary structure, and significantly decreased its ability to cause clinical symptoms of allergic diarrhea. The native OVA induced stronger allergic reaction and higher activities of serum mast cell protease-1 and enterocyte brush border membrane alkaline phosphatase. Heated-OVA stimulated higher level of IgG2a in sera and higher IFN-γ secretion by MLN and splenocytes. Digestion of heated-OVA led to different fragments compared to OVA and to loss of Foxp3+ cells induction ability *in vitro*. Distinct enzymatic digestion of heated-OVA compared to native OVA leads to appearance of different antigenic epitopes recognized by T and B lymphocytes and is reflected in changes in T cell subpopulations, cytokine production and specific antibody formation that support the shift towards Th1 response. As a consequence, allergy development is reduced but not completely abolished (Golias et al. 2012).

A part of the laboratory under leadership of **Igor Splichal** has been interested in an ontogeny of innate immune system mechanism (pig fetuses and gnotobiotic piglets) and a role of probiotics in induction of innate immune mechanisms for protection of gnotobiotic piglets against enteric infections.

E. coli Nissle 1917 (EcN) is a probiotic bacteria that modulate natural immunity of the host against infection with enteric pathogens (Trebichavsky et al. 2010). The piglets infected with enteric pathogens *E. coli* O55 and *S. Typhimurium* suffered from infection. DNA-binding nuclear protein high mobility group box 1 (HMGB1) was transcribed in the terminal ileum of gnotobiotic piglets constitutively, regardless of any bacterial presence. In contrast, the transcription of cytokines was upregulated by virulent bacteria. HMGB1, IL-8, and TNF-α level in the ileum were increased by both enteric pathogens, while IL-10 levels increased in *E. coli* O55-infected piglets only. HMGB1 significantly increased in the plasma of piglets infected with virulent *E. coli* only, but cytokine levels were in most cases increased by both virulent bacteria. HMGB1 and cytokine levels in ileum lavages and plasma of piglets colonized with probiotic *E. coli* remained comparable to those of the non-stimulated germ-free piglets (Splichalova et al. 2011a). The local and systemic expression of HMGB1, its relationship to the bacterial translocation, inflammatory cytokines, and clinical findings showed HMGB1 as a suitable marker of severity of enteric infections (Splichalova et al. 2012). EcN has been reference bacteria for a searching for possible probiotic bacterial strains that make possible a comparison of protection properties in a gnotobiotic infectious piglet model of enteric infections. The colonization, translocation and protective effect of porcine commensal bacteria *Bifidobacterium choerinum*, strain PR4 in infection with *S. Typhimurium* was compared with EcN bacterial interferences in *Salmonella* infection. In contrast to the piglets previously associated with EcN that thrived, the piglets previously associated with PR4 show symptoms of infection (increased local and systemic levels of inflammatory cytokines, diarrhea, anorexia) similar to germ-free piglets directly infected with *S. Typhimurium* (Splichalova et al. 2011b).

Based on metabolic profile of EcN and *E. coli* O55, a modified MacConkey agar was developed to discriminate between both strains on the same Petri dish to study bacterial interferences inside of *E. coli* species (Splichalova et al. 2014). This work was done in collaboration with Vojtech Rada (Czech University of Life Sciences, Prague, Czech Republic) and Ulrich Sonnenborn (Ardaypharm GmbH, Herdecke, Germany).

We collaborate in project developing the recombinant DNA-binding protein HMGB1 for diagnostic application (Principal investigator – Vojtech Vejvoda, Generi-Biotech s.r.o., Hradec Králové, Czech Republic). We collaborate in projects improving of bacterial detection, porcine model of sepsis and porcine model in gastroenterology (Rada et al. 2010, Rockova et al. 2011, Kieslichova et al. 2013). Manuscript under review in World Journal of Gastroenterology (Hucl T., Benes M., Kocik M., Splichalova A., Maluskova J., Krak M., Lanska V., Kieslichova E., Oliverius M., Spicak J.: Comparison of inflammatory response to transgastric and transcolonic NOTES, 2015).

In collaboration with Yoshihiro Muneta (National Institute of Animal Health, Tsukuba, Japan) we studied the development of allele-specific primer PCR for a swine TLR2 SNP and we compared their frequency among several pig breeds of Japan and the

Czech Republic (Muneta et al., 2012a). Second study was focused on allele-specific primer polymerase chain reaction for a single nucleotide polymorphism (C1205T) of swine Toll-like receptor 5 and comparison of the allelic frequency among several pig breeds in Japan and the Czech Republic (Muneta et al. 2012b).

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Molecular Biology and Immunology

2.1 Introduction

The Laboratory of Molecular Biology and Immunology (LaMBI) with its focus on molecular biology and relevant technology development has officially originated out of the Laboratory of Innate Immunity in 2014. In order to transform, over the past five years the laboratory has undergone major reform regarding research interests, as well as personnel restructuring. Recently, it consists of a **fairly young team** that has the prospects to be successful professionally, especially in the field of therapeutic biosensors. The change of research interest of the original laboratory arose from the need for molecular biology and biotechnology expertise within the Department of Immunology and Gnotobiology, for which Dr. Benson was specifically brought on board. Therefore, within the years 2010-2014, the **main outputs of LaMBI are comprised of immunology, as well as from the publication of new technology**.

2.2 Characteristics of the Main Outputs, 2010-2014

LaMBI has a substantial background in research of innate immune cells, which has been confirmed in several **publications that describe the involvement and signaling of NK and NKT cells in human diseases**.

In the field of immunology we have investigated the above mentioned cell populations during the development and the progression of cancer, rheumatoid arthritis, and problematic *in vitro* fertilization. Within this field **we have published 8 papers** in peer reviewed journals. Most have encompassed our research regarding the characterization of **the effects of synthetic glycodendrimers on immune cells** and the definition of **intracellular pathways** triggered by the application of synthetic glycoconjugates. Here, we will summarize the most important research results within this field.

In fresh **NK cells** and the **NK-92** permanent cell line, we observed a significant **down-regulation of glycosyltransferase** MGAT3 and MGAT5 **mRNA expression**, after stimulation with a synthetic glycoconjugate consisting of a calixarene core, linked with four N-acetyl-D-glucosamine moieties (GN4C). An excessive expression of the above mentioned glycosyltransferases is often impaired in cancer and results in the impairment of cancer cell recognition by the NK cells. Inhibition of glycosyltransferase expression triggered by GN4C correlated with an **improvement of NK cell effector functions**, as well as an increased susceptibility of tumor cells to the cytotoxicity of fresh NK cells or NK-92 cells. The functional activation of NK cells was accompanied by an increased mRNA expression of the NK activation receptor NKG2D, as well as an increased synthesis of IL-2, IFN-gamma, and tumor necrosis factor-alpha. Regarding cellular signaling, we found that GN4C **engaged PI3-kinase/ERK** but not phospholipase C-gamma/JNK pathways (*Benson et al. International Immunology, 2010*). Employing *in vivo* studies in a **mouse melanoma**

model we observed that **GN4C significantly reduced the tumor growth and prolonged the survival** of experimental animals. In parallel to GN4C, we also found that another synthetic glycoconjugate consisting of a polyamidoamine bone and eight N-Acetyl-D-glucosamine residues (**GN8P) promoted the response of tumor specific B-cells**. This B-cell response correlated with primary NK cell activation (*Hulíková et al. International Immunopharmacology 2011*). Each of the above mentioned results were found exclusively by our laboratory members. At this point we realized that the long term hypothesis suggesting **GlcNAc as a ligand for NK cell activation receptors such as NKR-P1 is fairly controversial**, and thus we performed experiments to reevaluate this hypothesis. This work has been done in collaboration with the Faculty of Science of Charles University where V. Grobárová, member of the laboratory, completed her doctoral thesis. LaMBI contributed by obtaining primary data for the gene expression array analysis, as well as the RT-PCR validation of the array results. In this project we observed no affinity between the neoglycoproteins and the NKR-P1A receptors from mouse or rat. Nevertheless, the polyamidoamine conjugate exhibited specific-binding to the NIK cells and the macrophages but NK cells. Taken together with a lack of serum parameters describing an anti-cancer response (in mouse melanoma and colon cancer models), **we suggested the indirect involvement of the NK cells in a GN8P-mediated immune response** (*Grobárová et al. Re-evaluation of the involvement of NK cells and C-type lectin-like NK receptors in modulation of immune responses by multivalent GlcNAc-terminated oligosaccharides. Immunology Letters 2013, 156(1-2):110-7).*

We have also studied the role of NK cells in the environment of autoimmune diseases, such as rheumatoid arthritis. Here we found the importance of a surface glycosylation that plays a crucial role in disease pathogenesis. First, we investigated the role of the NK cells and the CD161 receptor in human rheumatoid arthritis (RA). Compared to healthy controls, **NK cell cytotoxicity of RA patients was significantly increased** ($p<0.0001$). We demonstrated that **NK cells are able to respond to a mutated citrullinated vimentin** (MCV), a RA-specific autoantigen. The stimulation of the NK cells with the MCV **increased both PAD4 enzyme and CD161 mRNA expression**. The **synoviocytes of RA patients also exhibited MGAT5 glycosidase involvement in GlcNAc metabolism**. Thus, we provided new insight into the pathogenesis of RA and confirmed the involvement of surface glycosylation (*Richter et al. Clinical Immunology 2010).* This work was performed in a tight collaboration with a clinical department of the Rheumatology Institute (RI) in Prague. The clinicians from the RI were responsible for taking samples and maintaining a clinical parameters database. The next step in the research of NK cells' role in RA was represented by a **project of a collagen-induced mouse rheumatoid arthritis *in vivo***. This project has been performed in a collaboration with the National Institute of Public Heath in Prague enabling statistical analyses, and with the Laboratory of Immunotherapy (IMB, AS CR) that assisted with disease development and progression evaluations. Here we uncovered a **reduced inflammatory infiltration as well as a suppression of the T, B, and the antigen-presenting cells in the synovia after stimulation with the glycoconjugates GN8P and GN4C** mentioned earlier. Our results support the modern hypothesis of a glycobiological approach to the treatment of collagen-induced arthritis/rheumatoid arthritis (*Richter et al. Collagen-induced arthritis: severity and immune response attenuation using multivalent N-acetyl glucosamine. Clinical and Experimental Immunology 2014, 177 (1): 121-133).*

In order to transform its research focus, **LaMBI has introduced molecular biology expertise with a modern nanotechnology approach.** This primary work has been performed within a consortium of the European project, DINAMO (7FP EU), and here **we have established ties with other professionals in the field of nanomaterials.** These collaborations have enabled the lab to gain expertise in the nanodiamond (ND) handling necessary for their evaluation as biosensors. **We have already published our first manuscripts (total 3) in this field** in peer-reviewed journals and carry on the original DINAMO project with our Nanointegrace project of the Ministry of Education and the European Social Fund. Within the Nanointegrace project we have studied a key activity focused on the development and characterization of **nanodiamond biosensors based on an effector RNA**, aimed for diagnostic and therapeutic use in cancer patients. Regarding this topic, we have compiled **two publications pending in 2015**.

Our **initial contribution** to nanotechnology research (the first 3 published papers) has been **based on the characterization of biocompatibility and the visualization of nanoparticles** with various surface modifications. First, we have visualized the intracellular localization of fluorescent nanodiamonds. These NDs have been developed and characterized in the Institute of Physics AS CR and at the Faculty of Biomedical Engineering of the Czech Technical University. We have focused on this nanomaterial because of its great physical and biological properties. The uniqueness of such a diamond nanoparticle is in its stable fluorescence in a near red region, which is enabled by the presence of nitrogen vacancy (NV) centers inside a crystal grid. In two papers, we have presented a novel method for the monitoring of biochemical processes based on **color changes of the luminescence originated from the NV centers inside the nanodiamond particle**. We have compared basic surface modifications, and have found that in contrast to the surface oxidized nanodiamonds, the hydrogenation of the NDs led to a quenching of the luminescence emitted from the negatively charged NV centers and changed the intensity ratio between the negatively charged and the neutral NV centers. A similar change occurs after the binding of a biomolecule such as nucleic acid, which **identifies the nanodiamond as a suitable approach for development as a biosensor** *(Petráková V. et al. Advanced Functional Materials 2012; Kratochvílová I. et al. Magnetical and Optical Properties of Nanodiamonds Can Be Tuned by Particles Surface Chemistry: Theoretical and Experimental Study. J. Phys. Chem. C, On-line Oct. 2014)*.

The surface of these oxidized nanodiamonds can be easily modified with an anticancer drug such as daunorubicin. In our collaborations with Prof. Ho from Northwestern University in Chicago, LaMBI has studied the toxicity of a drug-functionalized nanodiamond applied to live cellular systems. In this project we demonstrated that the application of the anticancer drug daunorubicin bound to a nanodiamond exhibited lower therapeutic toxicity than the application of the daunorubicin alone. That observation indicated that **ND carriers improved drug tolerance and may serve as promising beneficial drug carriers** (*Moore L. et al. Nanoscale 2014).*

2.3 Established Partnerships and the Transfer of Technology

Our Laboratory of Molecular Biology and Immunology participated in an international research and an education program of the Czech Technical University focused on the development of nanoprobes for the non-invasive detection, targeting, and real-time monitoring of transformed cells. These tasks have

been solved in collaboration with the research teams of Prof. Ho (Northwestern University, Chicago, IL, USA), Prof. Neužil (Griffith University, Brisbane, Australia), Prof. Nesládek (Interuniversitair Microelectronica Centrum, Hasselt, Belgium), Prof. Wrachtrup (Stuttgart University, Stuttgart, Germany), Dr. Ledvina and Dr. Cígler (Institute of Organic Chemistry and Biochemistry, Prague), and Prof. Kruger (Julius Maximilian University, Wuerzburg, Germany). In 2012 LaMBI member V.Grobárová completed a **short-term stay in the laboratory of Prof. Ho to gain new expertise in nanodiamond handling**. In respect to the determination of therapeutic biomolecules, we cooperated with the Faculty of Medicine of the Charles University based in Pilsen and the Institute of Biology in Pilsen (Dr. Pešta, 2015 - pending publication regarding the involvement of microRNA-21 in prostate cancer patients). In the field of Immunology we collaborated with the National Institute of Public Health in Prague (Dr. Malý), the Institute of Rheumatology (Dr. Vencovský, Dr. Svobodová), two Faculty Hospitals of Charles University (2nd and 3rd; Dr. Vránová, Dr. Starec, Dr. Vinakurau), and the Czech Technical University (Prof. Rosina). **In the collaborations with Prof. Nesládek and Dr. Cígler we submitted a new proposal regarding the *in vivo* application of these therapeutic nanosensors. It has recently been approved for funding by the Ministry of Health** of the Czech Republic **from 2015**, and its research strategy is detailed in section 3.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Immunotherapy

T**he Laboratory of Immunotherapy (156) has been established** in the Institute of Microbiology of the ASCR, v.v.i. **in 2012**, after the reorganization of the Department of Immunology and Gnotobiology. The Laboratory, related to the Lab. 156 - Laboratory of Natural Cell Immunity, directed by Dr. Luca Vannucci from June 2009, was established to better develop the **line of research on immunology of the tumor microenvironment.** The new Laboratory of Immunotherapy appointed an almost completely **new team**. It was assembled during the 2012 and included: two senior scientists (**Dr. Luca Vannucci, MD, PhD, director** and **Dr. Petr Šíma, PhD, part times**), one post-doc/young researcher (**Dr. Jiří Křížan, PhD**), two PhD students (**Dmitry Stakheev**, from September 2012, and **Fabián Čaja**, from October 2012), one master degree student (**Lenka Rajsiglová** from September 2012, but who was present in the lab since January 2012 for training and developing her bachelor thesis); two technicians (**Lucie Kralová Duchacková** and **Eva Truxová**). Additionally, Petr Okenfuss, a master degree student from the Czech University of Life Sciences in Prague, was in our laboratory for training from November 2012 until the end of June 2014, actively participating to the experimental activities. He was recently replaced by a new master degree student from the Faculty of Natural Sciences of the Charles University in Prague, **Martina Fialková** (from November 2014), who will develop her experimental thesis in our lab under the supervision of Dr. Křížan with the counseling of Dr. Vannucci and Dr. Šíma. **Despite the age, we liked to maintain the additional collaboration of Dr. Šíma and Ms. Truxová (both already part of the previous laboratory) because their specific expertise, the first for his knowledge about natural products with potential immunomodulatory activity (e.g. glucans, nucleotides) and his skill in science popularization; the second, for her skills in laboratory animal care and manipulation**. According to its main composition, the Laboratory of Immunotherapy results to be **a very young laboratory** in which a young scientist and student at different level of their scientific evolution can find the **concrete occasion to test their capacities and develop in their knowledge and skills**. The work is planned by Dr. Vannucci in the view to **assist the progressive acquirement of confidence and responsibility of each element of his team in carrying on the experimental activity and developing their specific capacities and skills** within the various aspects of the experimental practice. The **team is international** (Czech, Italian, Slovak and Russian) and **adequately balanced in the genders and in the ages**, a fact that permits an **excellent dynamic in socialization, reciprocal collaboration and respect, favoring the adequate functionality of the laboratory activities and relationships**. Finally, the possibility to have **international guests** in the laboratory, either **internationally recognized scientists or researchers co-working even for extended periods**, permits comparisons, discussions and interactions that are useful for the progressive enhancement of both the quality of work and the use of English language (in the years visited our previous and new laboratory guests like Prof. Alberto Mantovani, Prof. Vincenzo Bronte, Prof. Viktor Umansky, Prof. Roni Apte, Prof. Antonio Gomez-Munoz, Prof. Adriana Albini). In the 2014 we also started a first attempt,

according to the financial possibilities, to send young members of the laboratory to autonomously participate to international congresses with a their work (e.g. Dr. Jiří Křížan and Fabián Čaja at the 2 nd. Meeting of Middle-European Societies for Immunology and Allergology. October 10-13. 2013. Opatija, Croatia) or visiting other laboratories (Dr. Křížan spent a short period on November 2014 in the laboratory of Prof. Giovanni Porta at the Department of Experimental and Clinical Biomedical Sciences, Faculty of Medicine of the University of Insubria, Varese, Italy, to process and test some of our experimental materials together with the local team, in the frame of a started collaboration).

Our research has the central point in the tumor microenvironment and how immunity, tumor and normal tissue interplay in its establishment and evolution. Looking from different viewpoints we try to define conditions and mechanisms suitable for interventions able to remodulating the altered immunity in the tumor microenvironment and rescuing an effective anticancer response. Inflammation and cancer is the base from which we have started and on which we are continuing our investigations, with a special focus on tissue remodeling. During the period 2012-2014 (but starting with various sets of experiments since 2008) we obtained some interesting results, part published and part under preparation for publication that we shortly summarize.

To better understand the influence of immune activation or inflammation in a tissue, we found optimal **the study of the bowel in a comparison between gnotobiologic (germ-free, GF) animals and conventionally reared animals (conventional, CV)**. We found that the presence of microflora produce an increase of proinflammatory cytokines in the colon mucosa of CV rats in comparison to GF rats (by ELISA and RT-PCR).

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This condition is associated to increase of TGFbeta in CV mucosa. Considering the importance of TGFbeta not only as regulatory molecule with inhibitory properties on the immune cells but also for its action on fibroblast in tissue remodeling, **we have investigated the collagen structure using the second harmonic generation technique with 2-photon confocal microscope** on fresh, unstained samples. **We have discovered significant difference in the collagen scaffold of the colon mucosa of healthy GF vs. CV rats,** with more elaborated architecture and higher accumulation in the CV rat colon. It is interesting that, looking to the structure of the GF rat mucosa after chronic inflammation, it appears to develop toward a “maturation” and thickening of the structure that is not becoming aberrant like in CV rats.

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We think these aspects to be related to the higher TGFbeta1 expression in CV rat colon consequent and in continuous presence of higher pro-inflammatory cytokine expression, **suggesting regulative network to maintain a protective threshold to immune activation in the CV mucosa**. Deregulation of this balanced network can lead to the pathological changes (fibrosis) produced by chronic inflammatory diseases and even in the altered inflammation developing during carcinogenesis and tumor development.

Microbiome can influence the structure of the bowel mucosa and its response to pathologic stimulation changing the threshold of responsiveness to and regulation of the inflammatory stimuli. Comparing the structural changes induced in GF animals by conventionalization, azoxymethane (AOM) and DSS induced carcinogenesis and

inflammation, **we discovered that the aseptic stimuli are able to induce highly dynamic and intensive remodeling of the collagen scaffold associated to expansion of mucosal lymphatic follicles.** This indicates the strict and reciprocally important relationship between immune cells, their activation and the surrounding structures. **The carcinogen produced comparable effects on structures and cell relationship than the other inflammatory stimuli (bacteria or DSS), confirming the involvement of inflammatory signals in cancer evolution and remodeling of the local microenvironment.** On these bases we induced carcinogenesis in CV animals or colitis or both together. The results obtained after long and repeated evaluation are summarized in the following picture that indicates a different role for IL6 in carcinogenesis than in chronic colitis. AOM carcinogenesis induces expression of IL6 that is increased when AOM is administered in the presence of DSS colitis.

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Consensually to both DSS and AOM pro-inflammatory effects, the **TGFbeta1 increases, with more evident progression when both inducers are associated. Effectively, more intense remodeling of the collagen scaffold and earlier onset of tumors are associated to these data (at 4 months from induction)**. Since the characteristics of different involvement of inflammatory cytokines in early period after the induction (1 month), we have started to investigate for other cytokines and cells possibly involved, like the Th17 cells, as well as for molecules involved in the stiffening of tissue and thickening of the collagen structures and their implication in controlling TNFbeta1 levels in the microenvironment (Caja F, Vannucci L. TGFβ: A player on multiple fronts in the tumor microenvironment. J Immunotoxicol. 2014 Aug 20:1-8. [Epub ahead of print] PubMed PMID: 25140864; Vannucci L. Stroma as an Active Player in the Development of the Tumor Microenvironment. Cancer Microenviron. 2014 Aug 9. [Epub ahead of print] PubMed PMID: 25106539). The final preparation and evaluation of these data was recently completed and a publications are under preparation.

Same collagen organization we have found in the mouse (GF and CV) and human colon samples from cancer patients (that overlap the rat CV colon mucosa alteration of collagen structure density induced by the experimental carcinogenesis).

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The IAA500200917 grant was a collaborative grant between our laboratory at the Institute of Microbiology, the Laboratory of the Genetics of Cancer at the Institute of Experimental Medicine of the ASCR, v. v. i. and the Department of general surgery of the Thomayer's Teaching Hospital of the 1st Medical faculty of the Charles University in Prague. Our Laboratory was coordinating the study (PI: Dr. Luca Vannucci) designing the experiments, following the preparation and performance of the experiments in vivo (induction of DSS colitis, AOM carcinogenesis), harvesting of the samples, immunological and microscopic evaluation of the samples. Dmitry Stakheev was involved in SHG imaging abd evaluation, becoming very skilled in confocal microscopy; Fabián Čaja was conducting the ELISA and WB evaluation, Dr. Křížan was performing PCR and FACS analysis and helping in the coordination of the experiments. The germ-free rats were obtained by another laboratory of the Institute of Microbiology: the Laboratory of Gnotobiology in Novy Hradek (CZ). Thanking a long lasting

collaboration with Dr. Renata Štepanková (since 2000) expert in GF rats rearing, Dr. Hana Kozaková (director of the Laboratory) and counseling of Prof. Helena Tlaskalová , emeritus, international expert on microbiota and intestinal chronic inflammation, groups of GF rats were prepared and reserved to our laboratory, eventually after having completed the protocol of induction. The team from the Institute of Experimental Medicine, leaded bz Dr. Pavel Vodicka, a recognized genetist in the study of colon carcinogentetic events, was participating to the harvesting of samples and performing numerous tests for evaluating possible genetic alterations in early phases of carcinogenesis and in the other induced conditions. No ras oncogene mutations were found while down-regulation of Apc and MMR genes resulted early associated to the carcinogenesis process induced by DSS+AOM and considered as a prerequisite for the development of colorectal carcinoma (CRC). **In this study were addressed for the first time early functional alterations of tumor suppressor genes with underlying epigenetic mechanisms** in experimentally induced CRC in rats (Polakova Vymetalkova V, Vannucci L, Korenkova V, Prochazka P, Slyskova J, Vodickova L, Rusnakova V, Bielik L, Burocziova M, Rossmann P, Vodicka P. Evaluation of tumor suppressor gene expressions and aberrant methylation in the colon of cancer-induced rats: a pilot study. Mol Biol Rep. 40(10):5921-9, 2013). The Department of Surgery, was furnishing human samples of colon mucosa from cancer patients. Samples were collected and frozen for further evaluation after initial controls about the general structure of the collagen structure, which resulted perfect homologue of the rat structure (as show above in Fig.6).

Another approach followed in our Laboratory, in the view of targeted intervention on the components of the tumor microenvironment that we will find more suitable and promising, has been the evaluation of melanoma targeting nanoparticles in the classical B16F10 mouse melanoma model. Thanking a cooperative grant with the Department of Biochemistry of the University " La Sapienza"in Rome and CNR of Rome, we have tested and contributed to better develop ferritin-based nanoparticles targeting the MC1R molecule, receptor for the melanoma stimulating hormone, highly expressed at the surface of human melanoma cells but also of the mouse melanoma cells. The nanovector (HFt-MSH-PEG), based on recombinant human protein ferritin (HFt), was functionalized with both melanoma-targeting melanoma stimulating hormone (□-MSH) and stabilizing poly(ethylene glycol) (PEG) molecules.

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After *in vitro* tests of selectivity and preliminary *in vivo* tests of biodistribution, with confocal evaluation in samples of freshly harvested organs of the localization of the nanoparticle labelled with rhodamine, we demonstrated the selectivity of HFt-MSH-PEG for melanoma. The results of this study were published on 2012 [Vannucci L, Falvo E, Fornara M, Di Micco P, Benada O, Krizan J, Svoboda J, Hulikova-Capkova K, Morea V, Boffi A, Ceci P. Selective targeting of melanoma by PEG-masked protein-based multifunctional nanoparticles. Int J Nanomedicine 7:1489-509, 2012 (9338 views on web version – with 2100 downloads – since March 2012) http://www.dovepress.com/selective-targeting-of-melanoma-by-peg-masked-protein-based-multifunct-peer-reviewed-article-IJN]

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To assess the potential use of HFt-MSH-PEG NPs as carriers for diagnostic and therapeutic agents, we investigated the *in vivo* behavior of these nanovectors compared with that of untargeted HFt-PEG NPs. Our constructs were detected in tumor-bearing mouse models

using two independent techniques; *ex vivo* whole-specimen confocal microscopy and *in vivo* magnetic resonance imaging/spectroscopy (MRI/MRS). We demonstrated that targeted HFt-NPs were able to accumulate selectively and persistently in primary melanoma with respect to other organs. In the melanoma tissue NPs were still present after 7 days while in the liver and in the spleen they were negligible (Fig. 8). Untargeted HFt-NPs also showed melanoma localization, but this was relatively less pronounced and disappeared more rapidly, indicating that active targeting contributes to melanoma localization. Accordingly, targeted HFt-based NPs localized at the melanoma level to a significantly larger extent than to a different type of tumor (adenocarcinoma) that does not express MC1R. Importantly, HFt-MSH-PEG NPs were able to target melanoma metastases, and may therefore be suitable carriers for in vivo delivery of diagnostic and/or therapeutic agents.

The results of the *in vivo* study were approved for publication in 2014 and published on January 2015 (**Vannucci L**, Falvo E, Failla CM, Carbo M, Fornara M, Canese R, Cecchetti S, **Rajsiglova L, Stakheev D**, **Krizan J,** Boffi A, Carpinelli G, Morea V, Ceci P: In vivo targeting of cutaneous melanoma using an MSH-engineered human protein cage bearing fluorophore and MRI tracers. Journal of Biomedical Nanotechnology 11(1):81-92, 2015 IF 7,578). The study was performed thanking the grant No. MFAG10545 of the Italian Association for Cancer Research (AIRC), Milan (IT) and the donations by ENI Czech Republic, Manghi Czech Republic s.r.o. Fund (CZ), Paul's Bohemia Trading s.r.o. (CZ) and Torino-Praga Invest s.r.o. (CZ). Our laboratory team was actively participating to the design of the experiments, the evolution of the nanoparticle (NP) characteristics and the performance of *in vivo* experiments with preparation of the tumor bearing mice, administration of the NP, sacrifice and sampling at time points, confocal study and analysis, preparation of the paper. Lenka Rajsiglova for the animal treatments and follow-up and Dmitry Stakheev for the confocal microscopy were particularly involved under the guide of Dr. Vannucci supported by Dr. Křížan. The PI's team was preparing the NPs, caring for all steps of their collection, purification, functionalization, as well as coordination of other groups for the biological *in vitro* study, histology, *in vivo* magnetic resonance evaluation of maghemite filled NP for evaluation of differences of distribution in the specific target (melanoma) vs. a breast adenocarcinoma in mouse model (the melanoma presented quite homogeneous localization, while the adenocarcimoma showed only limited vascular/perivascular localization).

The possibility to evidence ferromagnetic materials in the used models is promising for theranostic applications. Dr. Vannucci has a long experience (since 1992) on experimental microwave (MW) hyperthermia as an anticancer tool. On 2014 Dr. Vannucci has been approved as a local manager (together with Prof. Jan Vrba of the Faculty of Electro-technics of the Czech Technical University) of the COST Action TD1301 MiMed for the development of new diagnostic tools for breast cancer using MW. In the study in also included a therapeutic approach related to nanoconstructs to be used also as enhancers of altered tissue visibility. Our interest is to use a thermal modulation of the microenvironment to induce immune reactions able to modify the tumor microenvironment and/or facilitate eventual immunotherapeutic approach favoring tumor cell apoptosis and increase of oxygenation and molecule/drug/antibodies penetration by modifying the microcirculation and capillary permeability. Within a group of managers of the COST MiMed network, including Dr. Vannucci, a consortium was created for Horizon 2020 application in 2014. The first application was successfully approved, as was announced in February 2015, and the full

application was recently delivered for the second evaluation. Inside the project our laboratory will be involved in the animal tumor modelling, evaluation of biological and immunological effects of MW irradiation for imaging, and tissue analysis.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Photosynthesis - Algatech Center

In the research of efficiency of photosynthetic apparatus in various microorganisms with emphasis to antenna complexes and mechanisms of non-photochemical quenching we obtained the following results:

We showed that the pCO_2-dependent stimulation in organic carbon and nitrogen production was highest under low light. We attribute these responses to changes in the allocation of photosynthetic energy between carbon acquisition and the assimilation of carbon and nitrogen under elevated pCO2. We found that diazotrophic cyanobacterium Trichodesmium, which is source of more than 50% of biological nitrogen in the oligotrophic oceans, increases nitrogen fixation under increased CO_2 level. We showed that this stimulation in organic carbon and nitrogen production was highest under low light and we attributed them to changes in the allocation of photosynthetic energy between carbon acquisition and the assimilation of carbon and nitrogen under elevated pCO_2.. We also showed increased activity of NifH, PsbA, PsaC, AtpB a RbcL apoproteins without their increased abundance. The observed high flexibility in utilization of sources and energy in Trichodesmium is mediated by changes in redox state of photosynthetic electron chain and post-translational enzyme regulations. The results were obtained in collaboration with Institute of A. Wegenera in Bremerhavenu (SRN) and Bar-Ilan University in Israel, we did spectroscopic analysis of cultures, participated in joint experiments and analyzed the data (Kranz et al. Plant Physiol 154, 334, 2010 and Levitan et al. Plant Physiol 154, 346, 2010).

We described fast photoprotective nonphotochemical quenching (NPQ) in a new type of alga, Chromera velia, with a close evolutionary relation to apicomplexan parasite. We have identified that the photoprotection is enabled by a fast xanthophyll cycle and it is triggered by lumen acidification. The high concentration of violaxanthin, which we observed in C. velia, has therefore a dual role, it acts in light-harvesting and in photoprotection. The project was done exclusively in Třeboň (Kotabová et al. FEBS Lett 585, 1941, 2011).

We performed the first detailed analysis of photosynthetic efficiency and photoacclimation capacity of Chromera velia, a close relative to apicomplexan parasites with a functional photosynthetic plastid. We found that its photosynthesis is very efficient with the ability to acclimate to a wide range of irradiances. C. velia is able to significantly increase photosynthetic rates when grown under a light-dark cycle with sinusoidal changes in light intensity. All experiments and data analyses were performed in Třeboň (Quigg et al, PLoS ONE 7, e47036, 2012).

In collaboration with P.S.I. company (Brno) in the frame of joint EEA project (Norwegian funds) we developed a new type of bioreactor for cultivation of algae under precisely defined CO_2 concentrations (Utility model for bioreactor PMT1000).

We participated in identification of an effective photoprotective mechanism (reaction center-type nonphotochemical quenching) in Photosystem II (PSII) from extremophilic red algae (Krupnik et al. J. Biol. Chem. 2013). The obtained data indicate that PSII from this organism represents one of the most robust natural water-splitting complexes that can work under excess irradiance and that is useful for future construction of artificial systems mimicking photosynthesis. The key measurement of PSII antenna size was performed in Třeboň and these results were combined with biochemical isolation (by J. Kargul) and electron microscopy (in Boekema's laboratory) (Krupnik et al. J Biol Chem 288, 23529, 2013).

We identified a new type of photoprotective nonphotochemical quenching (NPQ) in cryptophyte algae. It is triggered by lumen acidification (stimulated mostly by linear electron flow) and it is situated in trans-membrane light-harvesting antennae (LhcR) and not in lumenal phycobiliproteins. These membrane antennae are characteristic by unique combination of pigments without any typical carotenoids usually involved in NPQ in higher plants. There is no light-induced xanthophyll cycle in this alga. The work was entirely done in Třeboň (Kaňa et al. PLoS ONE 7, e29700, 2012).

We proved that in cyanobacteria with phycobilisomes formed by rods and core, part of the fluorescence induction (the "S to M rise" in tens of seconds) is due to state 2 to state 1 transition. The pronounced SM rise reflects a protective mechanism for excess energy dissipation in cyanobacteria that are less efficient in other protective mechanisms, such as blue light induced non-photochemical quenching. All experiments and data analysis were performed in Třeboň and Třeboň team (5 of 7 authors) was leading the writing of the manuscript (Kaňa et al. BBA- Bioenerg 1817, 1237, 2012).

Using the novel single-cell approach of Fluorescence Kinetic Microscopy (FKM), we showed that mature heterocysts in the diazotrophic cyanobacterium Anabaena sp. strain PCC 7120 have intact and functional Photosystem II and strongly reduced PS II-associated antenna. This is in contrast to the current accepted view on the functional remodeling of photosynthetic membrane in heterocysts. This opens a new view on the bioenergetic processes active during nitrogen fixation **(**Ferimazova et al., Photosyn Res 116, 79-91, 2013).

We showed that phycobilisomes, pigment-protein complexes situated on thylakoid surface of red algae and cyanobacteria, are mobile in mesophylic but not in extremophilic strains. The immobility was rationalized by a strong protein-protein interaction between phycobilisome and photosystem(s). The mobility/immobility was reflected in a different regulation of excitation energy flow. The Třebon's team was involved in all physiological and microscopic measurements with initial assistance from experts at QMU. Třeboň group also participated in development of a new mathematical procedure for calculation of diffusion coefficient (together with Dr. Matonoha) (Kaňa et al. Plant Physiol, 165, 1618, 2014).

We analyzed in detail chromatic adaptation in C. velia cultured under blue or red light. Growth of C. velia under red light induced the accumulation of a novel light harvesting antenna complex with unusual spectroscopic properties. We showed that Red-LHC complex is assembled from a 17 kDa proteins, is connected to photosystem II and serves as an additional locus for non-photochemical quenching. The team from Třeboň performed most of the experiments (Kotabová et al. BBA-Bioenerg 1837, 734, 2014)

In the research of tetrapyrrole biosynthesis pathway we obtained the following results:

We have analyzed *Synechocystis* mutants lacking various parts of the C-terminal region of ferrochelatase, enzyme which synthesizes heme and has the C-terminus with putative chlorophyll-binding domain (CAB domain). Deletion of the CAB domain prevented the mutant strain from growing at higher light intensities and caused accumulation of chlorophyllide. We also demonstrated an essential role of the CAB domain for the ferrochelatase dimerization. Practically all laboratory work was done by R. Sobotka in Třeboň and partly during his postdoc stay in Neil Hunter's lab (Sheffield University), A. Wilde provided a construct for expression of tagged ferrochelatase (Sobotka et al. Plant Physiol 155, 1735, 2011).

We provided a non-phylogenetic evidence for Chromera velia close relationship to Apicomplexa. We showed that the heme pathway in C. velia highly resembles that in apicomplexan parasites, because it also synthetizes the first common precursor (aminolevulinate) in the mitochondrion. This is rather unique in phototroph making C. velia the only known phototroph able to synthesize chlorophyll from glycine instead of glutamate. The crucial biochemical measurements based on incorporation of ^{14}C-labelled amino-acids into chlorophyll molecules were performed in Třeboň under supervision by R. Sobotka (Kořený et al. Plant Cell 23, 3454, 2011).

We provided evidence that some aerobic organisms can live without heme, as is here the case of the protozoan Phytomonas. This is substantial, since so far heme was considered essential for all known forms of life. Moreover, this discovery may contribute to the development of more effective drugs against leishmaniasis, a serious tropical disease that causes the related parasite Leishmania, which has striking similarities with Phytomonas, when heme pathway in considered. The heme analyses performed by R. Sobotka provided key evidence that Phytomonas cells contain no heme molecules (Kořený et al. PNAS 109, 3808, 2012).

We showed that the Ycf54 protein forms a complex with the only known subunit of the oxidative Mg-protoporphyrin methylester cyclase enzyme. The Ycf54 has been shown to be essential for normal levels of cyclase activity and/or stability in the cyanobacterium Synechocystis 6803; the Ycf54 deficiency caused massive accumulation of the cyclase substrate and chlorosis. J. Kopečná and R. Sobotka performed phenotypic analyses of the mutant and the pigment analysis. R. Sobotka is corresponding author and wrote most of the paper (Hollingshead et al. J Biol Chem 287, 27823–27833, 2012).

In the attempt to to clarify the role of light and dark protochlorophyllide oxidoreductase (LPOR and DPOR) we assessed synthesis and accumulation of Chl-binding proteins in mutants of cyanobacterium Synechocystis PCC 6803 that either completely lack LPOR or possess low levels of the active enzyme due to its ectopic regulatable expression. In the LPOR-less mutant containing 20 % of the wild-type Chl level both Photosystem II (PSII) and Photosystem I (PSI) were reduced to the same degree. The phenotype of the LPOR-less mutant was comparable to the strain lacking DPOR that also contained less than 25 % of the wild-type level of PSII and PSI when cultivated in the dark. All work was done exclusively by members of the laboratory in Třeboň (Kopečná et al. Planta, 237, 497, 2013).

We investigated the presence of oxygen dependent and independent forms of oxidative cyclase among phototrophic Proteobacteria. This enzyme is the key enzymes of the bacteriochlorophyll biosynthesis pathway. The majority of purple non-sulfur bacteria contained both forms while the purple sulfur bacteria contained only the oxygen-independent form. Data suggest recruitment of the oxygen-dependent cyclase by purple non-sulfur bacteria very early during their evolution. The work was done entirely in Třeboň (Boldareva-Nuianzina et al. Appl Env Microbiol 79, 2596, 2013).

We were first to purify and characterize chlorophyll-synthase, an enzyme catalyzing the last step of chlorophyll biosynthesis pathway. Using a spectrum of biochemical methods we demonstrated that in Synechocystis it is a pigment-protein that associates with the high-light-inducible protein HliD, the PSII assembly factor Ycf39 and the Alb3/YidC insertase. This work provides the first evidence for the hitherto elusive link between chlorophyll and photosystem apoprotein synthesis. We performed majority of work, all pigment and protein analyses were performed in Třeboň and M. Linhartová contributed equally to J. Chidgey (Chidgey et al. Plant Cell 26, 1267, 2014).

In the research of biogenesis of photosynthetic apparatus with emphasis to Photosystem II assembly we obtained the following results:

We found that the carotenoid-less mutant of the the cyanobacterium *Synechocystis* is light sensitive and cannot assemble PSII core complexes while formation of Cyt b6f complex and PSI, which predominantly accumulated in the monomeric form, is not inhibited Collaborators constructed the mutants but the key analyses of photosynthetic complexes using spectroscopic and electrophoretic methods were performed in Trebon (Sozer et al. Plant Cel Physiol 51, 823, 2010).

We showed that the cells of the cyanobacterium Synechocystis acclimated to high irradiance exhibit markedly reduced content of photosystem I (PSI) but they grew much faster and synthesized significantly more chlorophyll (Chl). Interestingly, almost all labeled de novo Chl was localized in the trimeric PSI, whereas only a weak Chl labeling was found in Photosystem II (PSII). These results unexpectedly suggest that PSII subunits are mostly synthesized using ‘recycled’ Chl molecules. All work was done exclusively by members of the laboratory in Třeboň (Kopečná et al. Plant Physiol 158, 476, 2012).

We localized the Psb27 protein in photosystem II (PSII) complex in the cyanobacterium Synechocystis sp. PCC 6803. The protein was identified in associations with CP43 antenna. Unexpectedly, a small amount of Psb27 was also detected in a complex with Photosystem I. Participation of Psb27 in the PSII assembly process was proposed. The work was done almost exclusively by members of the laboratory in Třeboň. Collaborators from Imperial College provided the psb27 deletion strain and were involved in writing. P. Halada was involved in characterization of the CP43 cleavage by MS and J. Nickelsen provided two hybrid data (Komenda et al. Plant Physiol 158, 476, 2012).

We analyzed the structure of the cyanobacterial PSII auxiliary protein Psb28. Determination of the crystal structure of the protein Tlr0493 from the thermophilic cyanobacterium Thermosynecho-coccus elongatus at a resolution of 2.3Å together with analysis of Psb28 from wild type and mutant strains of Synechocystis 6803 suggested that Psb28 exists as a dimer in vivo. In contrast, the more distantly related Psb28-2 protein found in Synechocystis

6803 was detected as a monomer in vivo. Overall our data suggest that the dimer interface in the Psb28 crystal might be physiologically relevant. Třeboň team performed biochemical analyses of all Synechocystis strains used in the study and identified dimer of Psb28 and monomer of Psb28-2 homologue (Bialek et al. 2013, Photosynth Res. 117, 375-383, 2013).

In membranes of Chromera velia we found core photosynthesis proteins PsaA and AtpB broken into two fragments and this is the first report on such structure of photosystem I and ATP synthase in any organism. These fragments are independently transcribed, translated, and assembled into functional complexes. Transcription profiles supported expression of many other modified proteins. Canonical gene clusters and operons were fragmented and reshuffled into novel putative transcriptional units. The C. velia plastid genome was identified as linear-mapping, a unique state among all plastids. R. Sobotka and J. Komenda performed proteomic analysis of photosynthetic complexes using 2D electrophoresis providing key evidence that the central subunits of photosystem I and ATP are assembled from fragments (Janouškovec et al. Mol Biol Evol 30, 2447–2462, 2013).

We showed that the loss of photoautotrophy in the Synechocystis mutant lacking prepilin peptidase is caused by the toxicity of the unprocessed form (prepilin) of the pilin PilA1. The presence of the prepilin in membranes impaired the synthesis of Photosystem II subunits but not Photosystem I and induced the degradation of Sec translocons. These data suggest that the PilA1 prepilin interferes with the translocon machinery required for the synthesis of pilins and Photosystem II. It is the first reported link between synthesis of photosynthetic apparatus and pilins. The work was done entirely in Třeboň except mass-spectrometric analyses which were provided by P. Halada and T. Ječmen from Prague (Linhartová et al. Mol Microbiol 93, 1207, 2014).

We identified a novel chlorophyll and β-carotene binding protein complex in the cyanobacterium Synechocystis important for formation of the Photosystem II reaction center assembly complex. It was composed of putative short-chain dehydrogenase Ycf39 and two members of the high-light-inducible protein family. Data indicate a role of the complex in the delivery of chlorophyll to newly synthesized D1 protein. Sequence similarities suggest the presence of a related complex in chloroplasts. The project was almost exclusively in Třeboň, collaborators from Imperial College only provided Ycf39 deletion strain and were involved in writing, P. Halada from Prague performed the initial identification of the protein by mass spectrometry (Knoppová et al. Plant Cell 26, 1200, 2014).

In the research of protein quality control with emphasis to the role of FtsH proteases in cyanobacteria we obtained the following results:

We found that the FtsH2 protease encoded by the *slr0228* gene plays a major role in the degradation of both precursor and mature forms of the D1 protein, the key subunit of Photosystem II, following donor-side photoinhibition in the cyanobacterium *Synechocystis* sp. PCC 6803. The entire work was done in Trebon, collaborators provided several mutants and participated in writing (Komenda et al. BBA-Bioenerg 1797, 566–575, 2010).

We proved that three of four FtsH homologues in Synechocystis form a hetero-oligomeric complexes. We investigated the structure of the isolated FtsH2-GST/FtsH3 complex using transmission electron microscopy and single

particle analysis. The three-dimensional structural model revealed that the complex is hexameric and consists of alternating FtsH2/FtsH3 subunits. This is the first rigorous structural characterization of any photosynthetic FtsH complex. Members of the laboratory constructed and characterized strain with depleted essential FtsH3 protein contributing to the identification of the FtsH2/FtsH3 heterocomplex in Synechocystis, they also participated in the writing (Boehm et al. Plant Cell 24, 3669, 2012).

We showed that the heterocomplex of membrane FtsH proteases FtsH1/FtsH3 located in cytoplasm is involved in the acclimation of cells to iron deficiency via regulating the level of the Fur transcriptional repressor. Levels of the Fur protein declined in the WT but not in the FtsH3 or FtsH1 down-regulated strain while a mutant over-expressing FtsH1 showed reduced levels of Fur. The entire work was done in Třeboň, collaborators from Imperial College provided FtsH2 deletion strain and strains with GST-tagged FtsH and were involved in writing (Krynická et al. Mol Microbiol 94, 609, 2014).

In the research of aerobic anoxygenic photosynthetic bacteria we obtained the following results:

Special attention was payed to their genomics and to bioinformatic analyses of their genetic inventory and this was the topic of three following articles:

The genome of our isolate *Erythrobacter* sp. NAP1 was sequenced using the shotgun method by the J. Craig Venter Institute. The genome contains one continuous 38.9 kb long photosynthetic gene cluster, which contains a complete set of genes for bacteriochlorophyll biosynthesis and reaction center proteins. Strain NAP1 lacks genes of any CO2 fixation pathway which is consistent with its photoheterotrophic character (Koblížek et al J Bacteriol 193, 5881, 2011)

In anoxygenic phototrophs the genes encoding the photosynthetic apparatus are organized in a so-called photosynthetic gene cluster (PGC). In this study, the organization of PGCs was analyzed in ten AAP species belonging to orders Rhodobacterales, Sphingomonadales and to the NOR5/OM60 clade. While PGCs of all the analyzed species contained the same set of genes for bacteriochlorophyll synthesis and photosynthetic centers assembly, they differed largely in the carotenoid biosynthetic genes. (Zheng et al PLoS ONE 6, e25050, 2011).

The Roseobacter clade constitutes a large part of marine microbial communities. The origin of phototrophy in the clade was analysed from available genome sequences. The analyses suggest that the photosynthetic genes were not acquired via horizontal gene transfer, but rather all the Roseobacter species descend from ancient fully photosynthetic bacteria have lost some of the photosynthetic genes forming photoheterotrophic or eventually strictly heterotrophic species. (Koblížek et al. Advances Bot Res 66, 385, 2013)

The physiological and ecological studies are topics of the following articles:

The distribution of aerobic anoxygenic phototrophs (AAPs) was surveyed in various regions of the Mediterranean Sea in spring and summer. The AAP abundances increased with increasing trophic status and they made up on average 1-4% of total prokaryots in low nutrient areas, whereas in coastal and more productive areas 3-11% of total prokaryotes. The lower AAP abundances registered in the most oligotrophic waters suggest that they are rather poor competitors under nutrient limiting conditions. All measurements

and microscopic analyses except the microscopic counts from Famoso cruise were performed in Třeboň (Hojerová et al. Environ Microbiol 13, 2717, 2011).

We characterized growth of a phototrophic strain Erythrobacter sp. NAP1 in a carbon-limited chemostat regimen on various carbon sources under different irradiances. When grown in a light-dark cycle, these bacteria accumulated up to 110% more biomass in terms of carbon than dark cultures. Data showed that the additional energy from light allows the aerobic anoxygenic phototrophs to accumulate the supplied organic carbon which would otherwise be respired. The work was entirely done in Třeboň (Hauruseu and Koblížek, Appl Environ Microbiol 78: 7414, 2012).

We used infrared epifluorescence microscopy to follow temporal changes of AAPs in the alpine lake Gossenköllesee. AAPs abundance was low until mid July and reached a maximum by mid September. We also compared the studied lake with other mountain lakes located between 913 and 2799 m above sea level. The analysis of pufM diversity revealed that AAP communities inhabiting alpine lakes contain almost exclusively Sphinogomonadales species and are much more homogenous in comparison with low altitude lakes. Z. Cuperova performed the cell counts of AAP bacteria in the alpine lakes and analyzed their diversity using pufM clone libraries. Colleagues from Austria performed the seasonal study and performed the cell counts in Třeboň (Čuperová et al. Appl Env Microbiol 79, 6439, 2013).

We discovered of a novel phototrophic bacterium belonging to the phylum Gemmatimonadetes. This strain from a lake in the Gobi Desert contains photosynthetic genes originating from a horizontal gene transfer from purple phototrophic bacteria. The results not only describe a new phototrophic bacterial phylum, but also evidences a transfer of full photosynthetic ability between distant bacterial phyla. The work was done completely in Třeboň, except the fact that Dr. Feng from the University of Inner Mongolia performed the field sampling and sent us the water sample (Zeng et al. PNAS 111, 7795, 2014).

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Algal Biotechnology - Algatech Center

The research engage in our laboratory focuses on two main areas of interest: (i) the searching for bioactive secondary metabolites synthesized by microalgae and (ii) a bit more applied field of algal biotechnology. Within these two related areas we reach following results:

Bioactive secondary metabolites

We develop generally applicable platform for screening process including strains cultivation, extraction, purification, testing of biological activities and subsequent structure elucidation of an active microalgal secondary metabolites.
As an example is the HTS platform, based on AlphaLISA^{TM} technology, enabling to quantitatively identify anti-inflammatory compounds capable of reducing TNF-induced chemokine (IL-8, MCP-1) and adhesion molecule (ICAM-1) expression in human lung microvascular endothelial cells. The method value was demonstrated on cyanobacterial secondary metabolites screening.
Output: Journal of Photochemistry and Photobiology B: Biology, 18(I), 67-74, 2013.
Contribution of the group: The group members were responsible for cultivation of cyanobacterial strains and fractionation of secondary metabolites.
As the first we report the large scale screening on cytotoxic activity of terrestrial cyanobacteria. We have demonstrated frequent occurrence of cytotoxicity. The occurrence of cytotoxic strains was found to be dependent of the strain origin. According to the HPLC-MS/MS analyses only minority of observed compounds corresponded to known structures; thus, pointing to the high potential of terrestrial cyanobacteria in both pharmacology and biotechnology.
Output: Environmental Toxicology, 26 (4), pp. 345-358, 2011.
Contribution of the group: The group members were responsible for main experimental work including cultivation, extraction, cytotoxicity testing and HPLC-MS analyses as well as design of the experimental work and preparation of the manuscript. Co-authors from other institutes provided strains for the study.

In the last five year period an extensive screening of crude cyanobacterial extract has been performed by our screening platform. The studies had comprised over 200 cyanobacterial strains. The most effective crude extracts were selected based on the morphological changes resembling apoptosis and/or strong cytotoxic effect. These samples were subjected to an activity guided fractionation and several novel bioactive secondary metabolites has been isolated and characterized: **Nostotrebin 6**, a new polyphenolic compound with a fully substituted 2,2′-bis(cyclopent-4-en-1,3-dione) skeleton, was isolated from the cyanobacterial strain *Nostoc* sp. str. Lukešová 27/97. The compound is an *S*-parabolic *I*-parabolic noncompetitive inhibitor of acetylcholinesterase (IC_{50} = 5.5μM) and mixed inhibitor of butyrylcholinesterase (IC_{50} = 6.1–7.5μM).
Output: Journal of Enzyme Inhibition and Medicinal Chemistry, 25 (3), 414-420, 2010.
Contribution of the group: **The paper was designed, experiments performed and co-authored only by group members**, the co-authors participate on the HRMS and NMR measuring and **data analyses**.

We also prove the moderate cytotoxicity (IC_{50} = 12.2μM) of Nostotrebin 6 tested under in vitro conditions using mouse fibroblasts (BALB/c cells). It was found that this compound is capable of inducing both types of cell death, apoptosis and necrosis in a dose-dependent manner and antibiotic activity to some gram positive bacteria (MIC = 0.0156 $mg.ml^{-1}$).
Output: Molecules, 16 (5), pp. 4254-4263, 2011. Patent CZ304337.
Contribution of the group: The group member was responsible for cultivation of cyanobacterial strain producing Nostotrebin 6 and preparation of Nostotrebin 6 in sufficient purity and quantity.

Additional interesting feature of Nostotrebin 6 is their ability to create of nonconducting films which make uniform, defect-free and stable layers with permselective properties on gold and carbon surfaces under electrooxidation. The polymeric film can be used for electrode coating and thus preparation of an anti-interference barrier, for example, in dopamine microelectrode sensor construction. The results are the first evidence for the preparation of phenolic cyclopentenedione-based functional film.
Output: Electrochemistry Communications 38, 53–56, 2014. Patent CZ 304417.
Contribution of the group: The group member was responsible for cultivation of cyanobacterial strain producing Nostotrebin 6 and preparation of Nostotrebin 6 in sufficient purity and quantity.
For preparation of sufficient amount of more than 99 % pure Nostotrebin 6 from producing cyanobacterial strain *Nostoc* sp. str. Lukešová 27/97 we develop unique two-step operation high performance countercurrent chromatography (HPCCC) method based on a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (4:5:4:5, v/v/v/v).
Output: Molecules 19, 8773-8787, 2014.
Contribution of the group: The paper was designed, experiments and analyses performed, data analyzed and co-authored mainly by group members, the co-authors participate on the HPCCC method development.

Aeruginosin 865, a novel unusual glycosylated structural variant of known group of cyanobacterial peptides aeruginosins. The compound with the formula $C_{41}H_{64}N_6O_{14}$, and molecular weight of 864.9787 showed promising anti-inflammatory properties when tested by AlphaLISA essay for pro-inflammatory mediators IL-8 and ICAM. Moreover aeruginosin-865 did not show any cytotoxic effect in human fibroblasts and HeLa cells, which makes it an interesting immune-modulatory agent. In addition this compound has strong inhibition activity to serine proteases like trypsine, thrombine and elastase.
Output: Chem. BioChem., 14 (17), pp. 2329-2337, 2013.
Contribution of the group: The group members has performed isolation and purification of the compound in sufficient amounts for NMR and mass spectrometry measurements and also performed cytotoxicity tests as well as writes the manuscript with help of other co-authors.

Puwainaphycin F/G. We isolated and characterized two new cyanobacterial lipopeptides puwainaphycins (formula F: $C_{53}H_{87}N_{13}O_{15}$, M=1146.336; formula G: $C_{54}H_{89}N_{13}O_{15}$, M=1160.6672) and characterize their cytotoxic (mean IC_{50} = 2.2μM) effects in human cells in *vitro*. We have brought first evidence on membrane disrupting activity and potential toxicity of cyanobacterial lipopeptides. The Puwainahycin F/G cytotoxic effect was connected to intracellular calcium leakage and subsequent morpho-physiological changes leading to cell necrosis.
Output: Chem. Res. Toxicol., 25, 1203−1211, 2012.
Contribution of the group: The compound was discovered and purified by the group members. In addition the basic cytotoxicity test and IC_{50} values determination was done in our

laboratory only. Writing of the manuscript was co-ordinated by group members with help of other co-authors.
On the example of Puwainaphycin F/G we identify the first biosynthesis cluster of cyanobacterial β-amino lipopeptides and discuss the biosynthesis of lipopeptides in general. A combination of genome sequencing, bioinformatics and analytical chemistry analyses provides a powerful tool for the discovery of novel bioactive compounds, and new ways of environmental monitoring of unusual cyanotoxins.
Output: PLOS ONE, 9 (11) e111904, 2014.
Contribution of the group: **The paper was designed, experiments performed, data analyzed and co-authored only by group members.**

Muscotoxin A, B. We described two novel cyanobacterial metabolites (Muscotoxin A and B) isolated from soil cyanobacterium *Desmonostoc muscorum*. Muscotoxin A is a cyclic undeca-lipopeptide of sequence *cyclo*[Adhde^{1}-Gln^{2}-Gly^{3}-Pro^{4}-Phe^{5}-Ile^{6}-Ser^{7}-Dhb^{8}-Ser^{9}-Ile^{10}-Pro^{11}] containing lipophilic residue 3-amino-2,5-dihydroxydecanoic acid (Adhde), formula: $C_{58}H_{90}N_{12}O_{16}$ and M=1211.406. Proline at position 4 is substituted by rare γ-methylproline in Muscotoxin B. Muscotoxin A was cytotoxic to three mammalian cell lines (IC_{50} = 9.9-13 μM) inducing membrane damage, influx of calcium ions, mitochondria fragmentation and cell lysis. Our findings support the hypothesis that membrane permeabilisation might be a common toxicity mechanism of cyanobacterial lipopeptides.
Output: Chemical Research in Toxicology, in press.
Contribution of the group: The group members has performed cultivation, isolation and purification of the compound for NMR, mass spectrometry and chiral GC measurements and also performed cytotoxicity tests as well as writes the manuscript with help of other co-authors.

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Scytophycins. We develop method for analysis and structural elucidation of new members of the macrolide Scytophycins by HPLC-ESI-MS^n. The interpretation of fragmentation pattern of unknown Scytophyvin variants is based on "shifting" technique of measured mass spectra. The mixture of Scytophycins with strong antifungal and cytotoxic activity was isolated from cyanobacterium *Nostoc punctiforme*, str. Lukešová 5/96.
Output: Chemistry of Natural Compounds 49, 1170-1171, 2014**.**
Contribution of the group: **The paper was designed, experiments performed, data analyzed and co-authored only by group members.**

Algal biotechnology

Photosynthesis in microalgae mass cultures, maintenance and exploitation of mass cultures. An understanding of photosynthesis is fundamental for microalgal biotechnology to optimise growth of microalgal cultures. Various techniques of culture monitoring are described, as well as various laboratory and outdoor cultivation systems, biotechnologically important strains of microalgae, processing of microalgal biomass, microalgal products for nutrition, high-value and bioactive compounds, biofuels and ecological application of microalgal cultures.
Output: Journal of Industrial Microbiology & Biotechnology 37, 1307-1317, 2010.
Journal of Industrial Microbiology & Biotechnology 38, 307-317, 2011.
Eur. J. Phycology 47, 169–181, 2012.
Biomass Bioeng. 54, 115-122, 2013.

Aquat Biol 22, 95-110, 141-158, 2014.
Masojídek J, Torzillo G, Koblížek M (2013) „Photosynthesis in Microalgal Mass Culture“, in: *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, (editors: A.Richmond & Q. Hu). 2nd edition, Wiley-Blackwell, p. 21-36.
Masojídek J, Torzillo G (2014) Mass Cultivation of Freshwater Microalgae. *On-line database* Earth Systems and Environmental Sciences, Elsevier, *2nd edition, 13 p*.
Contribution of the group: The main role in all these studies was carried out by **group members.**

Changes of photosynthetic activity monitored by chlorophyll fluorescence techniques – correlation of fluorescence variables with growth and productivity. We elaborate monitoring methods of physiological and photobiochemical properties for the optimisation of the cultivation conditions for microalgae growth (temperature, pH, irradiance, addition of nutrients and CO_2). Chlorophyll fluorescence measurement has become widespread to monitor photosynthetic performance of microalgal mass cultures. Chl fluorescence variables can be related to changes of cultivation conditions, physiological status and growth of microalgal cultures for a given microalgal strain and cultivation system. We aimed to provide simple, practical guides to the use of chlorophyll fluorescence techniques for biotechnologists who wish to monitor changes of photosynthetic activity and optimize the growth of microalgal mass cultures.
Output: Aquat Biol 22, 124-140, 2014.
Masojídek J, Vonshak A, Torzillo G (2011) Chlorophyll Fluorescence Applications in Microalgal Mass Cultures. In: Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications (eds. DJ Suggett, O Prášil, MA Borowitzka). Springer, Dordrecht. pp. 277-292.
Contribution of the group: The main role in all these studies was carried out by **group members**, in collaboration with partners in Italy and Spain.

Application of microalgal biomass as feed supplement. We elaborate the cultivation process for certain strains of fresh-water microalgae for the targeted production of the biomass enhanced with the required valuable substances (chlorophylls, carotenoids, lipids and some organically bound chemical microelements). Biomass of microalgae enriched in bioactive compounds (chlorophyll, carotenoids, lipids) or enriched of organically bound elements (selenium) was used as a feed additive in fish aquaculture.
Output: Aquaculture Nutrition 17, 278-286, 2011.
Aquaculture Res 44, 157-159, 2012.
J Appl Ichthyol 29, 172-180, 193-199, 2013.
Neuroendocrinology Letters 35 (suppl. 2), 71–80, 2014.
Contribution of the group: The equal contributions in these studies were made by **group members** and the University of South Bohemia.

The design and construction of novel cultivation system - Think Layer Cascade. Outdoor thin-layer cascade units used in Třeboň represent highly productive system for microalgae cultivation due to their unique design. Its high productivity is achieved by the interplay among cell density, light path and culture turbulence that provide suitable average cell irradiance for high photosynthetic activity. New thing layer cascade unit is designed as a pilot production module with area 100 m^2 and total working volume of 750 L. Basic part of this unit is sole surface made of a steel construction and stainless steel sheets. Algal suspension flows along slope surface in a uniform layer. Suspension is delivered to the highest point by circulation pump from a collecting tank which is located at the lowest point of the module. The suspension is aerated by CO_2 in the collecting tank.

Output: Utility design UV 27021, Patent CZ 304988.
Masojídek J, Sergejevová M, Malapascua JR, Kopecký J (2014) Thin-layer systems for mass cultivation of microalgae: flat panels and sloping cascades. In: *Algal Biorefinery, vol. 2* (editors: R. Bajpai, A. Prokop, M. Zappi) (under revision)
Contribution of the group: The contributions in these developments **and co-authored was only by group members.**

Pilot Production Unit with Artificial Illumination. Pilot production unit with artificial illumination consist of a closed vessel of working volume of 100 L internally illuminated using light-emitting diodes (LED). Lighting can be regulated by cell density so as to avoid photoinhibition in diluted culture or photolimitation in dense culture. Photobioreactor has effective mixing either by aeration or by mechanical agitation of the culture suspension so to ensure uniform illumination of the microalgal cells, temperature control, supply of nutrients and gas exchange (CO_2/O_2).
Output: Utility design UV 027242.
Sergejevová M, Malapascua JR, Kopecký J, Masojídek J (2014) Photobioreactors with internal illumination. In: *Algal Biorefinery, vol. 2* (editors: R. Bajpai, A. Prokop, M. Zappi) (under revision).
Contribution of the group: The main contribution to these studies was made by **group members only**.

Research Report of the team in the period 2010–2014

Institute	Institute of Microbiology of the CAS, v. v. i.

Scientific team	Laboratory of Cell Cycles of Algae - Algatech Center

The Laboratory of Cell Cycles of Algae has been involved in the study of green algae physiology, particularly their cell cycle regulation for more than 40 years. In recent years, the research team consists of three senior scientists supervising a group of postdocs, PhD and undergraduate students. Their work is supported by 2-3 experienced technicians. Until early 2013 the gender ratio of the team was strictly biased towards women who represented more than 80 % of the personnel; since 2013 the gender ratio has been becoming more balanced with women representing 70 % at the end of 2014 and less than 60 % at the beginning of 2015. The age structure suggests a bias towards younger team. In fact, two senior researchers in their early forties are supported by an advice and help from a partially retired founder of the laboratory. The postdocs and students represent the age group between 25 to 40, the technicians age range from less than 25 till 60 years old.

Below is outlined the multiple fields that have been covered by research of the laboratory in the past years. The main research directions include the study of molecular mechanisms regulating the algal cell cycle progression, study of different environmental factors affects on algal growth and study of accumulation of high-energy reserve compounds in algae. Whilst seemingly unrelated, all research is related to our main expertise, the multiple fission cell cycle of green algae. We take advantage of the extremely well characterized growth conditions of synchronous cultures to study various effects, treatments and growth conditions to better understand the physiology of algae. The algal unit in Trebon was founded more than fifty years ago with a single goal to understand the algal physiology in order to use algae as food or feed replacements/supplements. Currently, the view of the best use of algae has shifted to their usage for pharmaceutical, nutraceutical and/or biofuel production but the need to understand the physiology in order to maximize the production stays valid. Indeed, the study of cell cycle led to understanding how are produced as well as how are spent high-energy reserve compounds enabling thus over-production of starch and lipid by algal cells.

Cell cycle regulation in algae

The green algae dividing by multiple fission have been the premium model organisms of the laboratory. In contrast to most eukaryotic organisms dividing into two daughter cells, some algae can divide into more than thousand of daughter cells within single cell cycle while still following the same rules as in other cell cycles. Due to the autotrophic growth and the fact that DNA replication and nuclear and cellular divisions can occur in dark, the growth and cell cycle processes can be separated by transfer into dark. Thus the green algae can be easily synchronized by alternating light and dark regime leading to production of highly synchronous cultures where all cells are at the same stage of the cell cycle. Such cultures represent one of the best characterized growth/culture systems. Since the cells can be dividing in dark, in nature during night, there has been a long-term controversial whether the division is gated by circadian clock to happen only in dark or the cell cycle is circadian clock

independent. In a set of two papers (Vítová *et al.*, 2011a, b) we analyzed under very wide growth conditions the effect of light and temperature on the growth and division of model green alga *Chlamydomonas reinhardtii*. In accordance with previous authors, we found in certain relatively narrow range of conditions when the cell cycle was indeed happening approximately every 24 hours. However, we identified a wide range of conditions, when the cell division was not regulated by any timers including circadian ones, since the division occurred as often as every 10 hours or as rarely as every 73 hours for several cycles. Moreover, the experiments with differing temperatures proved that the cell cycle duration is not temperature compensated as is expected for a circadian timer regulated process. Taken together the data indicate there is no endogenous timer regulating the cell cycle progression in *Chlamydomonas reinhardtii* and the cell cycle is solely controlled by growth conditions set by combination of light, temperature and concentration of carbon dioxide. The research was done in our laboratory; our Japanese collaborator enabled us to re-test our conclusions on different cultivation set up. Thank to our interaction with our Japanese colleagues, we also started collaboration with another Japanese group working on different model alga, multicellular marine alga *Ulva compressa* (Kuwano *et al.*, 2014). Experience with this for us so far unknown organism was interesting and we obliged by testing the growth in our cultivation system and sharing our cell cycle expertise.

In different project, we have been trying to understand how the cell cycle progression is coordinated with response to DNA damage (Hlavová *et al.*, 2011). Response to DNA damage requires a block of cell cycle in order to ensure no cell cycle progression until DNA is repaired. While extensively studied in mammals and yeast the mechanisms of DNA damage response are only very little characterized in plants cells and even less in algae. DNA damage is usually activating two types of checkpoints, one during S phase and second one in G2 phase preventing mitosis of damaged DNA. During the multiple fission cell cycle of the green alga *Scenedesmus quadricauda,* there is multiple DNA replication steps followed by multiple G2 steps. Surprisingly, the algae respond differently to the same double stranded breaks DNA damage depending on the time of application. Induction of double stranded breaks early during the cell cycle delayed S phase and lead to permanent G2. In contrast, the same treatment during G2 phase did not cause cell cycle arrest suggesting a specific time window during multiple fission cell cycle when DNA damage checkpoint is not operating. The data suggest an evolutionary interesting alga-specific mechanism for coordination between DNA damage response and cell cycle progression.

Given the current increased interest in green algae study and our expertise in study of multiple fission cell cycle, we have been repeatedly invited to review the topic. Our motivation behind the review writing was to make available to the reader the outcome of more than sixty years of research of this fascinating cell cycle mechanism. As well as to highlight the advantages of such cell cycle organization for cell cycle study: high level of natural synchrony, clear relationship between cell cycle and cell growth and wide range of possible cell cycle alternations (Bišová and Zachleder, 2014; Zachleder *et al*., 2015).

1) **Hlavová, M.; Čížková, M.; Vítová, M.; Bišová, K.** ✉**; Zachleder, V**. 2011. DNA damage during G2 phase does not affect cell cycle progression of the green alga *Scenedesmus quadricauda.* **PLoS ONE** 6(5) e 19623. I.F. = 3.534

2) Kuwano, K.; Abe, N.; Nishi, Y.; Seno, H.; Nishihara, G.N.; Masafumi, I**.; Zachleder, V.** 2014**.** Growth and cell cycle of *Ulva compressa* (Ulvophyceae) under LED illumination. **J Phycol,** 50: 744-752. I.F. = 2.529
3) **Vítová, M.; Bišová, K.; Umysová, D.; Hlavová, M.; Zachleder, V.✉; Čížková, M.** 2011°. *Chlamydomonas reinhardtii*: Duration of its cell cycle and phases at growth rates affected by light intensity. **Planta** 233: 75–86. I.F. = 3.357
4) **Vítová, M.; Bišová, K.; Umysová, D.; Hlavová, M.; Zachleder, V.✉; Čížková, M.** 2011b**.** *Chlamydomonas reinhardtii*: Duration of its cell cycle and phases at growth rates affected by temperature. **Planta** 234: 599-608. I.F. = 3.357

Reviews:

5) **Bišová, K.✉; Zachleder, V.** 2014. Cell cycle regulation in green algae dividing by multiple fission. **J Exp Bot** 65: 2585-2602. I.F. = 5.24
6) **Zachleder, V**.✉; **Bišová, M.; Vítová, M.** 2015. Cell cycle of microalgae. In: **Microalgae Physiology.** Developments in Applied Phycology' Series (Borowitzka, M.; Beardall, J., Raven, J. eds) Springer, in press.

Accumulation of energy reserves in algae

The most of green algae (Chlorophyta) can produce both starch and lipids but overproduction of one or another type is distinct in different species or strains and can be regulated by growth conditions. In most of the "starch strains" starch production is inducible. However, lipid production is inducible only in some strains ("lipid strains"). This area of research has been recently for us the most productive one. We analyzed different algal strains for their ability to produce and/or over-produce starch and/or lipids. In the initial report (Brányiková *et al.*, 2011), we identified two important conditions for over-production of starch in green alga *Chlorella vulgaris*. The most important factor affecting the rate of starch synthesis and accumulation is mean light intensity resulting from a combination of biomass concentration and incident light intensity. Another important factor is the phase of the cell cycle. The starch is produced during the growth phase of the cell cycle but it is consumed by cell division even on light thus varying between

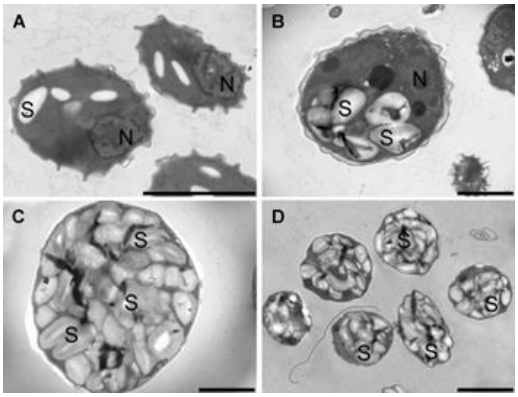

Fig. 1 Electron microscopic photographs of daughter (**A**) and mother (**B**) cells of green alga *Chlorella* grown in complete mineral medium, in the presence of cycloheximide (1 mg/L) (**C**), and in sulfur limiting medium (**D**). N, nucleus; S, starch granules. Bars: panels A–C 2 µm; bar panel D 5 µm.

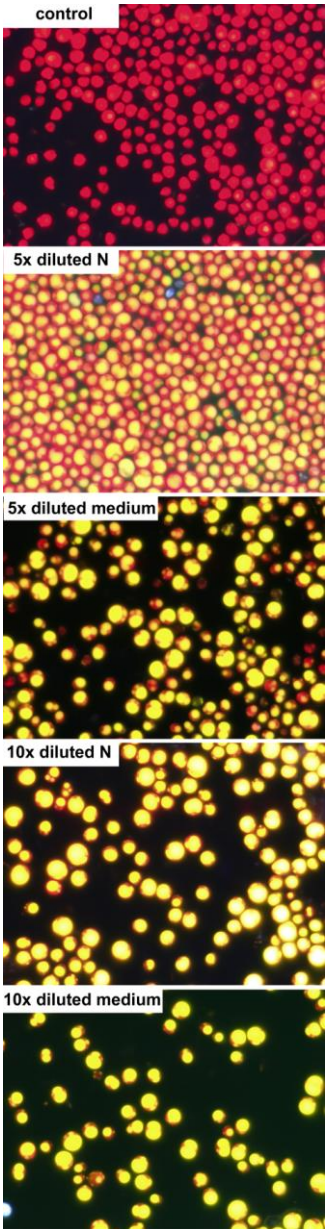

Fig. 2 Fluorescence micrographs of green alga *Parachlorella kessleri* cells grown for 13 days in full medium (control), medium containing 20% (5x diluted) or 10% (10x diluted) of nitrogen, medium diluted 5x or 10x by distilled water (5x diluted, 10x diluted). Neutral lipids in lipid bodies are visualized by Nile Red, red - autofluorescence of chloroplast, yellow - lipid bodies

45% of dry weight to about 4% for cells dividing in dark. To overproduce starch, we tested in laboratory conditions different treatments that would suppress cell division events, but not to disturb synthesis of starch in the chloroplast (Fig. 1). The most successful treatment, sulfur starvation, we also tested in open outdoor scaled-up thin-layer photobioreactor. The sulfur starvation proved to be efficient in starch overproduction since it lead to increase of starch content up to 50% of dry weight. This is one of the highest yields reached so far. Research was done in collaboration with Institute of Chemical Technology through a work of shared PhD student and training of master student. The expertise came from our laboratory and the work was performed there. In a sequel to this work we analyzed over-expression of lipids in different algal strain *Parachlorella kessleri* (Li *et al.*, 2013). In this microalga, nutrient starvation will also stop the cell cycle progression without affecting the chloroplast synthesis. The starvation initially led to production of starch but later the lipids started to be over-produced. For the over-production of lipids the most effective treatments were nitrogen starvation or dilution of the entire growth medium leading to accumulation of up to 60% of dry weight in the form of lipids (Fig. 2). Again, we were able to reproduce the laboratory results in open outdoor scaled-up thin-layer photobioreactor with lipid content reaching up to 25% of dry weight. The expertise of the laboratory in algal cultivation and production of different energy compounds was combined with expertise of Japanese colleagues in fluorescent microscopy and that of colleagues in Institute of Botany CAS on lipid analysis. The fact that both starch and lipids were produced in time dependent manner after nutrient starvation drew our attention to the coordination between synthesis and degradation of both high energy compounds (Fernandes *et al.*, 2013). The results indicated that the prolonged starvation lead to chlorophyll and starch degradation and to storage lipid over-production. Interestingly, starch was not used for storage lipid synthesis but the cellular lipids were. During recovery in full medium, cells divided, even in dark, while spending both starch and lipids as energy and carbon source. The work started as collaboration with Japanese group on the changes of energy compounds made during starvation of green algae, where our expertise in algal cultivation was exploited. Most of the cultivation work was done in our laboratory in collaboration with students from the Portugal group. Analyses of lipids during starvation were done in collaboration with P. Přibyl in Institute of Botany CAS.

Due to the overlapping expertise between our group, the group at University of Tokyo and a group at Institute of Botany we also participated in four other reports analyzing different aspects of starch and lipid accumulation and their coordination in different algal species (Přibyl *et al*., 2012; Mizuno *et al.*, 2013; Přibyl *et al.*, 2013; Takeshita *et al.*, 2014). For the collaborative papers we provided our expertise in algal cultivation and lipid and starch over-production.

Thanks to our experience and expertise in the field of energy reserves production we were also invited to summarize and compare recent development in this fast evolving field (Přibyl *et al.*, 2014; Zachleder *et al.*, 2014).

1) **Brányiková, I.**; Maršálková, B.; **Doucha, J.;** Brányik, T.; **Bišová, K.; Zachleder, V.** ✉; **Vítová, V.** 2011. Microalgae - novel highly-efficient starch producers. **Biotechnol Bioeng** 108: 766-776. I.F. = 4.164
2) **Fernandes, B.**; Teixeira, J.; Dragone, G.; Vicente, A.A.; Kawano, S.; **Bišová, K.;** Přibyl, P.; **Zachleder, V.** ✉; **Vítová, M**. 2013. Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga *Parachlorella kessleri*. **Bioresour Technol** 144: 268-274. I.F. = 5.039

3) **Li, X.;** Přibyl, P.; **Bišová, K.;** Kawano, S.; Cepák, V.; **Zachleder, V.**✉; **Čížková, M.; Brányiková, I.; Vítová, M.** 2013**.** The microalga *Parachlorella kessleri* – a novel highly efficient lipid producer. **Biotechnol Bioeng** 110: 97–107. I.F. = 4.164
4) Mizuno, Y.; Sato, A.; Watanabe, K.; Hirata, A.; Takeshita, T.; Ota, S.; Sato, N.; **Zachleder, V.**; Tsuzuki, M.; Kawano, S. 2013. Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. **Bioresour Technol** 129: 150–155. I.F. = 5.039
5) Přibyl, P.; Cepák, V.; **Zachleder, V**. 2012. Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris.* **Appl Microbiol Biotechnol** 94: 549–561. I.F. = 3.811
6) Přibyl, P.; Cepák, V.; **Zachleder, V.** 2013. Production of lipids and formation and mobilization of lipids bodies on *Chlorella vulgaris.* **J Appl Phycol** 25: 545–553. I.F. = 2.492
7) Takeshita, T.; Ota, S.; Yamazaki, T.; Hirata, A.; **Zachleder, V.**; Kawano, S. 2014. Starch and lipid accumulation in eight strains of six *Chlorella* species under comparatively high light intensity and aeration culture conditions. **Bioresour Technol** 158: 128-134. I.F. = 5.039

Reviews:

8) Přibyl, P. ✉; Cepák, V.; **Zachleder, V.** 2014. Oil overproduction by means of algae. In: **Algal biorefineries**. **Volume 1** (Bajpai, R.K.; Prokop A.; Zappi, M. eds) Springer Science+Business Media Dordrecht. pp. 241-273.
9) **Zachleder, V.** ✉; **Brányiková, I**. 2014. Starch overproduction by means of algae. In: **Algal biorefineries**. **Volume 1** R.K.; Prokop A.; Zappi, M. eds) Springer Science+Business Media Dordrecht. pp. 217-240.

Effect of selenium and rare earth metals

The effect of different metals on algal growth has been one of the long term projects in the laboratory. We have been studying the effect of cadmium, selenium and most recently also rare earth elements (REEs). Selenium is an essential element in many organisms but at high concentration it can also be a dangerous toxin. We have selected different strains of the green alga *Scenedesmus quadricauda* resistant to high concentrations of either selenate, selenite or both compounds. The strains allowed us to characterize the response of the alga to toxic concentrations of selenium. The function of selenium in an organism is mediated mostly by selenoproteins including potent anti-oxidative protein, glutathione peroxidase. The activity of glutathione peroxidase as selenium protecting enzyme in different strains reflected their resistance to different selenium compounds being high in the sensitive strains and low in the resistant strains. The selenium application also lead to ultrastructural, resistance dependent, changes in the chloroplast indicating chloroplast is one of the target organelles. For a biotechnology related research, the resistant strains were grown in an outdoor-photobioreactor in the presence of high concentration of selenium which was up taken into the cells. The selenium enriched cells were then used by our collaborators to support chicken diet and analyze its effect. The algal biomass increased not only the selenium concentration in the chicken muscle but also improved the oxidative stability of the meat suggesting benefits of using selenium enriched algae in chicken diet (Skřivan et al., 2010).

Recently, we have become interested in the effect of REEs on the algal growth. Similarly to selenium, the REEs have different effects depending on the concentrations. It was suggested that the effect of REEs could be mediated by their

ability to replace essential elements such as calcium. Indeed, we were able to prove that under calcium dependent conditions REEs can replace calcium and thus improve algal survival. This suggests lanthanides can replace essentials elements, but their effects on microalgae depend on stress and the nutritional state of the microalgae (Goecke *et al.*, 2015a). The effect of both selenium and REEs on the algal growth and physiology as well as the possible exploitation of algae for selenium and REEs recycling or remediation has been discussed in two recent reviews (Vítová *et al.*, 2015; Goecke *et al.*, 2015b).

1) **Goecke F.** ✉**;** Jerez C.; **Zachleder V.;** Figueroa F.L.; **Bišová K.;** Rezanka T.; **Vítová M**. 2015a. Use of lanthanides to alleviate the effects of metal ion-deficiency in *Desmodesmus quadricauda* (Sphaeropleales, Chlorophyta). **Front Microbiol** 6:2. I. F. = 3.941
2) Skřivan M.; Skřivanová V.; Dlouhá G.; **Brányiková I.; Zachleder V.; Vítová M.** 2010. The use of selenium-enriched alga *Scenedesmus quadricauda* in a chicken diet. **Czech J Anim Sci** 55:565–571
3) **Vítová, M.; Bišová, K.; Hlavová, M.; Zachleder, V.**✉; Rucki, M.; **Čížková, M.** 2011**.** Glutathione peroxidase activity in the selenium-treated alga *Scenedesmus quadricauda*. **Aquatic Toxicol** 102: 87–94. I.F. = 3.513

Reviews:

4) **Goetzke, F.; Zachleder, V.** ✉**; Vítová, M**. 2015b. Rare earth elements and algae: physiological effects, biorefinery and recycling. In: **Algal biorefineries. Volume 2** (Bajpai, R.K.; Prokop A.; Zappi, M. eds) Springer Science+Business Media Dordrecht, in press.
5) **Vítová, M.; Bišová, K.; Doucha, J.; Zachleder, V.** ✉ 2015. Selenium resistant and selenium enriched algae and their applications. In: **Algal biorefineries. Volume 2** (Bajpai, R.K.; Prokop A.; Zappi, M. eds) Springer Science+Business Media Dordrecht, in press.

Algal cultivation and nutrition

This research area has been in the past years represented mostly by invited reviews, we wrote alone or in collaboration with our colleagues. The reviews summarize different aspect of algal cultivation. They discuss how to optimize outdoor autotrophic algal cultivation by changing growth conditions such as light, temperature, mixing, carbon dioxide, oxygen and nutrition (Doucha and Lívanský, 2014). The effect of nutrient bioavailability as well as other environmental factors (light, pH, salinity) is further discussed elsewhere (Procházková *et al*., 2014). Changes in the nutrient composition are the cost effective strategy to induce shifts in biomass composition in order to achieve over-production of metabolites such as lipids, polysaccharides and pigments. The best practices in heterotrophic cultivation of algae were summarized in collaboration with colleagues from ZHAW, Switzerland, who are experts in heterotrophic cultivation (Bumbak *et al.*, 2011). Although the production of algae by heterotrophic cultivation usually provides high yields it should be kept in mind that not all algal strains are suitable for heterotrophic cultivation, and some of them require specific chemical composition of mineral growth media. Finally, we summarized how can be algal biomass, specifically that of genus *Chlorella*, used in animal nutrition (Kotrbáček *et al.*, 2015). *Chlorella* biomass improves animal growth and performance due to the presence of pigments, antioxidants, provitamins, vitamins and a growth substance known as the Chlorella Growth Factor (CGF). The *Chlorella* biomass can be also used as a carrier of organically bound selenium and

iodine. Generally speaking *Chlorella* biomass is a cost effective way of increasing the value of animal products for human consumption.

In the research paper, we combined our expertise in algal cultivation with that of experts in biogas production and co-generation (Doušková et al., 2010). We tested and experimentally verified in semi-industrial scale that algae can be cultivated on flue gas. Biogas from agricultural waste (distillery stillage) was used for co-generation of electricity and heat; the algal cultivation exploited electricity and heat for maintaining optimal growth conditions. Furthermore, the algae spent and, thus remediated carbon dioxide, either in biogas or in flue gas from co-generation unit. The "waste" carbon dioxide supported algal growth same as food-grade one.

1) **Doušková I.** ✉**;** Kaštánek F.; Maléterová Y.; Kaštánek P.; **Doucha J.; Zachleder V**. 2010. Utilization of distillery stillage for energy generation and concurrent production of valuable microalgal biomass in the sequence: biogas-cogeneration-microalgae-products. **Energy Convers Mngm** 51, 606-611. I.F. = 3.590

Reviews:

2) Bumbak, F.; Cook, S.; **Zachleder, V.**; Hauser, S.; Kovar, K. 2011. Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. **Appl Microbiol Biotechnol** 91: 31-46. I.F. = 3.811
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4) Kotrbáček, V.; Doubek, J.; **Doucha J.**✉ 2015. The chlorococcalean alga *Chlorella* in animal nutrition: a review. **J Appl Phycol** DOI 10.1007/s10811-014-0516-y. I.F. = 2. 492
5) Procházková, G.; Brányiková, I.; **Zachleder, V.;** Brányik, T. 2014. Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. **J Appl Phycol** 26: 1359–1377. I.F. = 2.492