Description of the main research directions investigated by the institute

IMIC is heterogeneous both in terms of the topics studied and in terms of the methodology used in the experiments, which is considered an advantage in today's world full of surprises and new challenges.

The main research areas of IMIC are biochemistry, physiology, molecular genetics and ecology of microorganism (bacteria, yeasts, filamentous fungi and algae) and immunology. Within these areas, regulatory mechanisms of cells in controlling growth, division and differentiation, synthesis of biologically active substances and enzymes, photosynthetic systems, mechanisms of DNA transfer, mRNA transcription and modification, regulation of protein synthesis, and degradation activities of microorganisms are all studied in great detail; as well as the topics of microbial contribution to the functioning of ecosystems, response of microbes to environmental conditions and general questions of ecology and biogeography are applied. Equally important are related immunological topics related to the response of animals to pathogenic microorganisms, to the mechanisms of acquired immunity and to the study of the influence of mucosal and intestinal microflora on the physiological processes of animals, including humans.

As of 31 December 2019, IMIC has 24 scientific laboratories and three service centers (DNA Sequencing Center, Cytometry and Microscopy Center, and Biotechnology Hall). In addition to the main campus in Prague 4 - Krč, IMIC also has 4 research facilities outside Prague:

1) BIOCEV Center (Vestec, Central Bohemian Region)
2) Algatech Center (Opatovice pond, Třeboň, South Bohemian Region)
3) Center for Nanobiology and Structural Biology (Nové Hrady, South Bohemian Region)
4) Laboratory of Gnotobiology (Nový Hrádek, East Bohemian Region)

IMIC research laboratories are separated into seven major categories of research:

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

Microbiology labs focus on research of molecular biology and genetics of prokaryotic microorganisms. They explore regulations of gene expression, effects of internal and

external conditions on cell functions, as well as molecular principles of bacterial pathogenicity.

Biology of the Cell and Bioinformatics labs concentrate on research of molecular biology and genetics of eukaryotic microorganisms and cells. They investigate regulation of gene expression, cell differentiation, and effect of internal and external conditions on cell functions, mechanisms of cell aging, and significance of cytoskeleton functions. Associated computer modeling of biological processes in the cell provides bioinformatics support for the experimental systems of the study.

Structural Biology laboratories combine three analytical tools: mass spectrometry, nuclear magnetic resonance spectroscopy and microscopy for characterization of molecular structures of selected subjects of study (such as various compounds, proteins, etc.).

Biogenesis labs study physiology and genetics of mycelial actinomycetes producing secondary metabolites and genetics, physiology and biotechnology of filamentous fungi. Other projects include exploration of antibiotic resistance of bacteria, biotransformation of natural compounds and enzyme technologies.

Ecology research program covers the ecology, physiology and biochemistry of microorganisms in their environments, especially in soils, plant litter, decomposing wood or in association with plants. It includes complex physiological, biochemical and genetic characterization of fungal enzyme systems capable of biodegradation of pollutants such as aromatic hydrocarbons, but also a general view of microbial involvement in ecosystem-level processes and the global ecology and biogeography of microorganisms. The interactions between symbiotic and pathogenic fungi and bacteria, their hosts and organic matter in soil are other important topics of study.

Immunology labs study the origin and development of immune response, functional characterization of components of the immune system and regulation of immune response. IMIC immunological researchers have made important breakthroughs in the diagnosis and treatment of cancers and autoimmune diseases.

The results obtained by all these laboratories open many promising possibilities for future industrial and biomedical applications.

During the evaluation period 2015-2019, the research portfolio of IMIC changed to reflect developments in microbiology and related fields of science. Historically, IMIC consisted of four main sectors whose research profiles did not change for a long period of time and for various reasons did not allow for dynamic changes in the focus of laboratories organized within these sectors. This changed in 2015 when the sectors were dissolved, allowing regrouping/refocusing of laboratories according to the actual

needs of PIs of individual laboratories, making the collaborations between research groups within the Institute much easier.

Announcement of new EU programs for research funding in 2016 prompted us to modernize the concept of IMIC research at that time. The newly proposed concept (designated MIC21) consisted of three pillars: (i) studies of the molecular architecture of the cell, focused on revealing new factors and mechanisms involved in functioning of the cell; (ii) studies of the microbiome, focused on discovering the basic principles of microbial communities in their natural environments; and iii) studies of pathogen-host interactions aimed at understanding their interplay from both prokaryotic and eukaryotic perspectives. Although the “MIC21” project was not funded in the end, its preparations stimulated intensive interactions between the majority of IMIC laboratories resulting in an establishment of the Core Facility for Bioinformatics (completed in 2019), which supports studies in the three main IMIC research directions.

i) The first pillar is the knowledge of the mechanisms of origin and possible therapies of serious human diseases, based on the analysis of important molecular processes taking place at the level of the single cell, the whole cell population and entire organism. Therefore, both prokaryotic (bacteria) and eukaryotic model systems (yeast, filamentous fungi, plant and mammalian cells) are analyzed at IMIC in response to changes in their living environment. These studies are focused on elucidation of detailed molecular mechanisms of regulation at the level of cell or the cell population (eg DNA replication, structure and function of RNA polymerase complex, translation, intracellular signaling). Results of these studies are then expected to be used in biotechnological applications (e.g. preparation of enzymes or metabolites with new chemical properties or atypical activities).

ii) The second pillar employs understanding the functioning of microbial communities and their interactions with the environment, plants and animals, how that plays a key role in finding solutions to existing human problems (such as the development and functioning of ecosystems in the context of global climate change) and their response to environmental threats. Methodologically, the possibility to effectively study the microbial interactome was opened with the advent of effective technologies of parallel sequencing, the development and application of high-throughput methods of instrumental analysis and relevant bioinformatics tools. Identifying global trends in research on microbial communities in soils and resulting complex environmental processes has significant impacts on agriculture and the environment at local and global levels.

iii) Studies of pathogen-host interactions and related immunological issues form the third important pillar of research at IMIC. The parallel of the soil ecosystem with the habitat of the intestinal mucosa is obvious; both ecosystems are characterized by a complex microbiome, where interactions occur between microorganisms and higher organisms. In this context, the topics of microorganism-host interactions in specific

models of clinically important pathogens (e.g., *Bordetella pertussis*, *Streptococcus pneumoniae*) are also addressed at IMIC. As the composition of the intestinal microflora significantly affects the response of the human body to immunotherapy, IMIC is also involved in analysis of the importance of the human microbiome in the study of tumor immunology.

Due to the specific method of subsidized financing of science by the government of the Czech Republic, all institutes of the Czech Academy of Sciences operate with budgets covering only basic salaries and operating costs. Therefore, the research directions of IMIC are mainly driven by the ability of PIs and the institute's management to acquire financial support for selected scientific topics on a project-oriented funding basis. Since the IMIC scientific portfolio includes well-recognized topics and its management has long-term experience in the application for and management of all types of grant projects, the institute's PIs are quite successful in obtaining grant funds from both national and international providers. This includes highly competitive funding schemes such as the ERC Synergy project, four Czech Science Foundation EXPRO projects, and four Czech Science Foundation Junior Star projects. Therefore, it is not surprising that a high number of top quality publications, including 21 highly cited papers (~50% more than in 2010-2014) were published during the previous evaluation period (2015-2019), highlighting the quality of the research IMIC conducts. In addition to its basic research activities, IMIC also has its own economic activities primarily focused on contract research and production in the fields of biology, chemistry and medical sciences. As dictated by the Czech law, their volume cannot exceed 4% of the total financial turnover of the entire institution. Finally, it is needless to say that IMIC has all relevant authorizations necessary for its numerous experimental activities; e.g. for work with GMOs, laboratory animals and radioactive material.

In conclusion, the Institute of Microbiology of CAS (IMIC) is an internationally competitive institution, in some fields considered a European leader and, as such, has guaranteed prospects for future growth.

Research activity and characterisation of the main scientific results

Our Lab is the world´s biggest centre for the ergot evolution study (M. Kolařík, Pešicová, M. Kostovčík, M. Chudíčková, E. Stodůlková, M. Flieger).

Ergot fungi are grass pathogens famous for their toxicity. Twenty six new *Claviceps* species, half of the known diversity, was described in our lab. The laboratory has the world´s biggest and most complete culture collection of ergot fungi, Culture Collection of Clavicipitaceae (CCC, 14000 strains) which serve as an international platform for the research of ergot biology.

Our revision of the agriculturally important species *C. purpurea* fundamentally changed the understanding of its biology (Pažoutová et al. 2015). This ergot species was considered as generalists, with extremely broad host range. We showed, that *C. purpurea* is a complex of four species, differing by their host range and we provided the formal names for that species. We defined host spectrum of the rye infecting species, *C. purpurea* sensu stricto, what will have impact on the biological protection against this toxicogenic fungus. This highly cited study becomes a gold standard for studies dealing with species delamination in ergot fungi and laid the foundations for the study of *C. purpurea* species group taxonomy and biology.

We continued in the description of ergot species diversity from Africa (van der Linde et al. 2016). Africa is home to numerous invasive grasses which destroyed natural grass ecosystems over the world. It was also shown that as a result, their specific ergot species expanded their host range. A crucial step in the monitoring of ergot host ranges, geographical distribution, and possible invasions is the characterisation of their host spectrum on native African grasses. In this study, we described five new toxicogenic species of ergot, incl. new potentially invasive ones.

We also firstly reconstructed the evolutionary history of the whole genus (Pešicová et al. 2018). We showed the presence of four biologically unique and deeply diverging groups, described as a new sections. Among them, only the section *Claviceps*, the group of rye ergot (*C. purpurea*) is typical by its high toxicity a broad host range. This lineage is further specific by the presence of so far neglected toxins, which are often more abundant than the well-known ergot alkaloids. Other newly erected sections are much less toxic and have narrow host range. The study is a starting point for study of ergot evolution. As a further step, we plan to use genomic data (first genomes published in Wingfiled et al. 2018) for more detail reconstruction of character evolution in *Claviceps*.

Ergot fungi are known for their production of ergot alkaloids and the great agricultural impact they have on both cereal crop and farm animal production. We showed that the content of so far ignored Ergochrome toxins (i.e. secalonic acid A-C) is comparable to the content of alkaloids in ergot and they can contribute to the overall ergot toxicity (Flieger et al. 2019). This study is the first to address the need of further study of neglected ergochromes and their role in acute and chronic ergot poisoning.

Selected outputs 2015-2019. All but one (Wingfield et al. 2018) studies were done almost exclusively by the Lab 112 members, which are also first and corresponding authors. Members from the outside of the lab provided some fungal specimens (P. Šrůtka, K. Broders, S. Wyka, EJ van der Linde), chemical analyses (Novák P, Man P, Kuzma M, Cvak L) and biological activity assays (J. Černý, Grobárová V). In case of

the study of Wingfield et al. (2018), M. Kolařík provided fungal strains and commented the data and manuscript draft.

1) **Pazoutova S**, **Pesicova K, Chudickova M**, Srutka P, **Kolarik M** (2015) Delimitation of cryptic species inside *Claviceps purpurea*. Fungal Biology 119: 7-26.
2) **Píchová K, Pažoutová S, Kostovčík M, Chudíčková M, Stodůlková** E, Novák P, **Flieger M**, van der Linde E, **Kolařík M** (2018) Evolutionary history of ergot with a new infrageneric classification (Hypocreales: Clavicipitaceae: *Claviceps*). Mol Phylogen Evol 123: 3-87.
3) van der Linde EJ, **Pešicová K, Pažoutová S, Stodůlková E, Flieger M, Kolařík M** (2016) Ergot species of the *Claviceps purpurea* group from South Africa. Fungal Biology 120: 917-30.
4) **Flieger M**, **Stodůlková E**, Wyka SA, Černý J, Grobárová V, **Píchová K**, Novák P, Man P, Kuzma M, Cvak L, Broders KM, **Kolařík M.** (2019 Ergochromes: heretofore neglected side of ergot toxicity. Toxins 11: 439.
5) **Kolařík M** (2015) New combinations for ergot species described under their anamorphic names by S. Pažoutová and colleagues. Czech Mycology 67: 135-136
6) Wingfield BD, Liu M, Nguyen HDT, Lane FA, Morgan SW, De Vos L, Wilken PM, Duong TA, Aylward J, Coetzee MPA, Dadej K, De Beer ZW, Findlay W, Havenga M, **Kolařík M**, et al. (2018) Nine draft genome sequences of *Claviceps purpurea* s.lat., including *C. arundinis*, *C. humidiphila*, and *C*. cf. *spartinae*, pseudomolecules for the pitch canker pathogen *Fusarium circinatum*, draft genome of *Davidsoniella eucalypti, Grosmannia galeiformis, Quambalaria eucalypti*, and *Teratosphaeria destructans*. IMA Fungus 9:401-418

Our results opened the new doors in the understanding of insect-fungal symbiosis. Harwood infesting bark beetles were long time ignored by the researchers. We showed that *Geosmithia* moulds are regular and often dominant and host specific symbionts of many bark and ambrosia beetles worldwide. Most of the known *Geosmithia* diversity was described in our lab including an invasive, tree killing fungus *Geosmithia morbida*. These fungi, together with another bark beetle symbiont, *Quambalaria cyanescens*, are producers of new biologically active compounds including patented compound with anticancer activity. Mutualistic fungi are not toxic to their animal host, but have to compete with other fungi and bacteria, what make them ideal target for antimicrobial drug discovery. Thus our interest in bioprospecting is focused to the secondary metabolites of little explored and new fungal lineages living in obligate mutualism with insects.

During the evaluated period we studied *Geosmithia* species across the western and eastern USA, to (i) provide baseline data on taxonomy of *Geosmithia* and vector specificity; (ii) investigate the subcortical beetle fauna for alternative vectors of the invasive *G. morbida*; and (iii) interpret the community composition of this region within the emerging global biogeography of *Geosmithia* (Kolařík et al. 2017, Huang et al. 2019). That studies are the starting points for the *Geosmithia* study in the USA. We also contributed to the understanding of symbiosis fidelity and its evolution in ambrosia beetles (Kostovcik et al. 2015) and we collaborated on the review of the current knowledge about the biology of tree killing European spruce bark beetle (Biedermann et al. 2019). We also continued in the description of the unknown diversity of bark beetle associated fungi and bacteria (Menéndez et al. 2015, Pepori et al. 2015, Saati-

Santamaría et al. 2018, Huang et al. 2018). Many of that species has great biotechnological potential (Fabryová et al. 2018, Saati-Santamaría et al. 2018), including production of a new metabolites, quambalarines, with anticancer activity (Stodůlková et al. 2015), which mode of action at the molecular level was further elucidated (Grobárová et al. 2016, Vališ et al. 2017) We identified the key adaptive traits in *Geosmithia* and traced the virulence factors of *G. morbida* (Veselská et Kolařík 2015, Veselská et al. 2019).

Selected outputs 2015-2019. The team members were first or/and corresponding authors in most cases (Stodůlková et al. 2015, Kostovcik et al. 2015, Veselská et Kolařík 2015, Menéndez et al. 2015, Kolařík et al. 2017, Saati-Santamaría et al. 2018, Fabryová et al. 2018, Veselská et al. 2019). In other studies we provided significant number of strains, molecular data or/and species descriptions and we collaborated on manuscript preparation (Pepori et al. 2015, Huang et al. 2018, Huang et al. 2019). In case of Grobárová et al. (2016) and Vališ et al. (2017) we stimulated the whole study and provided tested chemicals. In case of Biedermann et al. (2019) we participated on the writing the parts related with fungal symbioses and entomopathogens.

1) **Stodůlková E**, Císařová I, **Kolařík M, Chudíčková M**, Novák P, Man, M. Kuzma, B. Pavlů, J. Černý and **M. Flieger** (2015) Biologically active metabolites produced by the basidiomycete *Quambalaria cyanescens*. PLoS ONE 10(2): e0118913. doi:10.1371/journal.pone.0118913
2) **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
3) **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.
4) Pepori AL, **Kolařík M**, Bettini PP, Vettraino AM, Santini A (2015) Morphological and molecular characterisation of Geosmithia species on European elms. Fungal Biology. 119: 1063-1074.
5) Menéndez E, Ramírez-Bahena MH**, Fabryová A**, Igual JM, Benada O, Mateos PF, Peix A, **Kolařík M, García-Fraile P** (2015) *Pseudomonas coleopterorum* sp. nov., a cellulase producing bacterium isolated from the bark beetle *Hylesinus fraxini*. International Journal of Systematic and Evolutionary Microbiology. 65:2852-2858.
6) Grobárová V, Vališ K, Talacko P, Pavlů B, Hernychová L, Nováková J, **Stodůlková E**, **Flieger M**, Novák P, Černý J (2016) Quambalarine B, a Secondary Metabolite from *Quambalaria cyanescens* with Potential Anticancer Properties. Journal of Natural Products 79: 2304-2314
7) **Kolařík M**, Hulcr J, Tisserat N, De Beer W, **Kostovčík M**, Kolaříková Z, Seybold SJ, Rizzo DM (2017) Geosmithia associated with bark beetles and woodborers in the western USA: taxonomic diversity and vector specificity. Mycologia 109:185-199
8) Vališ K, Grobárová V, Hernychová L, Bugáňová M, Kavan D, Kalous M, Černý J, Stodůlková E, Kuzma M, Flieger M, J. Černý, P. Novák (2017) Reprogramming of leukemic cell metabolism through the naphthoquinonic compound Quambalarine B. Oncotarget 8:103137
9) Huang Y-T, **Kolarik M**, Kasson M, Hulcr J (2018) Two new *Geosmithia* species in *G. pallida* species complex from bark beetles in eastern USA. Mycologia, 109:5, 790-803.

10) **Fabryová A, Kostovčík M**, Díez-Méndez A, Jiménez-Gómez A, Celador-Lera L, Saati-Santamaría Z, Sechovcová H, Menéndez E, **Kolařik M, García-Fraile** P (2018) On the bright side of a forest pest-the metabolic potential of bark beetles' bacterial associates. Science of The Total Environment 619-620:9-17
11) Saati-Santamaría Z, López-Mondéjar R, Jiménez-Gómez A, Díez-Méndez A, Větrovský T, Igual JM, Velázquez E**, Kolarik M**, Rivas R, **García-Fraile P** (2018) Discovery of phloeophagus beetles as a source of *Pseudomonas* strains that produce potentially new bioactive substances and description of *Pseudomonas bohemica* sp. nov. Frontiers in Microbiology 9
12) **Veselská T,** Skelton J**, Kostovčík M**, Hulcr J, Baldrian P, **Chudíčková M**, Cajthaml T, **Vojtová T, Garcia-Fraile P, Kolařík M** (2019) Adaptive traits of bark and ambrosia beetle-associated fungi. Fungal Ecology 41: 165-176.
13) Huang, Y.-T., J. Skelton, A. J. Johnson**, M. Kolařík** and J. Hulcr (2019). *Geosmithia* species in southeastern USA and their affinity to beetle vectors and tree hosts. Fungal Ecology 39: 168-183.
14) Biedermann PH, Müller J, Grégoire J-C, Gruppe A, Hagge J, Hammerbacher A, Hofstetter RW, Kandasamy D, **Kolarik M, Kostovcik M** (2019) Bark Beetle Population Dynamics in the Anthropocene: Challenges and Solutions. Trends in ecology & evolution, 10: 914-924.

We follow the philosophy that researchers should help to make the life better. Our interest in human and plant pathogenic moulds of the *Aspergillus* genus resulted in the description of more than 50 new species, including new pathogens and important toxin producers. Large survey of dermatophyte diversity and taxonomy resulted in new diagnostic schemes, update of the epidemiologic situation and taxonomic novelties. The research on the bat killing fungus (*Pseudogymnoascus detructans*) identified the new virulence factors and contributed to understanding of its migration history.

Bats and cave environment

The long-time aim of our team is to describe pathogenic mechanism standing behind the mycotic disease of hibernating bats known as White nose syndrome. This disease has the fundamental impact on the Northern America bat population. We compared virulent and non-virulent strains of the etiologic agent and identified siderophores and vitamin B2 hyperaccumulation as the main virulence factors. Virulence factors revealing is the key step in the control of this severe disease (Flieger et al. 2016). Our team also contributed to the description of the disease areal and its spreading to the American continent (Zukal et al. 2016, Bandouchova et al. 2015, Pikula et al. 2017). Other activity involves description of the little known diversity of cave microbiota (García-Fraile et al. 2015, Nováková et al. 2017) including description of the new fungal genus *Myotisia cremea* (Crous et al. 2017).

Selected outputs 2015-2019.

We were first or/and corresponding authors in the study of Garcia-Fraile et al. (2015), Flieger et al. (2016) and Nováková et al. (2017). For the study of Crous et al. (2017) we contributed by the complex taxonomic description of *Myotisia cremea*. In other studies, we were responsible for the cultivation and molecular genetic identification of the *Pseudogymnoascus destructans* strains.

1) **Garcia-Fraile, P**, **Chudickova, M**, Benada, O, Pikula, J and **Kolarik, M** (2015) Serratia myotis sp. nov. and Serratia vespertilionis sp. nov., isolated from bats

hibernating in caves, International Journal of Systematic and Evolutionary Microbiology, 65: 90-94.
2) Bandouchova H, Bartonicka T, Berkova H, Brichta J, Cerny J, Kovacova V, **Kolarik M**, Koellner B, Kulich P, Martinkova N, Rehak Z, Turner GG., Zukal J, Pikula J (2015) Pseudogymnoascus destructans: Evidence of virulent skin invasion for bats under natural conditions, Europe. Transboundary and Emerging Diseases 62: 1-5.
3) Zukal J, Bandouchova H, Brichta J, **Cmokova A**, Jaron KS, **Kolarik M**, Kovacova V, Kubatova A, **Novakova A**, Orlov O, Pikula J, Presetnik P, Suba J, Zahradnikova A, Martinkova N (2016) White-nose syndrome without borders: Pseudogymnoascus destructans infection tolerated in Europe and Palearctic Asia but not in North America. Scientific Reports 6
4) **Flieger M**, Bandouchova H, Cerny J, Chudíčková M, **Kolarik M,** Kovacova V, Martínková N, Novák P, Šebesta O, **Stodůlková E**., J. Pikula (2016) Vitamin B2 as a virulence factor in Pseudogymnoascus destructans skin infection. Scientific Reports 6: 33200
5) Crous P, Wingfield M, Burgess T, Hardy GS, Barber P, Alvarado P, Barnes C, Buchanan P, Heykoop M, Moreno G et al. (2017) Fungal Planet description sheets: 558–624. Persoonia-Molecular Phylogeny and Evolution of Fungi 38:240-384
6)
7) Pikula J, Amelon SK, Bandouchova H, Bartonička T, Berkova H, Brichta J, Hooper S, Kokurewicz T, **Kolarik M**, Köllner B (2017) White-nose syndrome pathology grading in Nearctic and Palearctic bats. PLoS ONE 12:e0180435
8) **Nováková A, Hubka V, Valinová Š, Kolařík M**, Hillebrand-Voiculescu AM (2017) Cultivable microscopic fungi from an underground chemosynthesis-based ecosystem: a preliminary study. Folia Microbiologica: 1-13

Aspergillus moulds

Species of *Aspergillus* are moulds with great importance for human and animal health, agriculture and biotechnology. Our team focuses on the study of *Aspergillus* diversity, taxonomy, ecology, epidemiology and clinical importance. We described over 50 new species, including important human pathogens and we proposed new scheme for the species delamination. Outputs has a high relevance for the agriculture, food industry, biotechnology and human medicine.

Aspergillus section Flavipedes contains species found worldwide in soils and rhizospheres, indoor and cave environments, as endophytes, food contaminants and occasionally as human pathogens. They produce many extensively studied bioactive secondary metabolites and biotechnologically relevant enzymes. We revised, using polyphasic approach taxonomy of the section Flavipedes which includes three known and seven new species. In addition, *Aspergillus* section Jani was newly established (Hubka et al. 2015). Next studies solved taxonomy of section Nidulantes, what resulted in description of new sterigmatocystin-producing species (Hubka et al. 2016a). Further, we revised sections Petersoni (newly described) (Jurjević et al. 2016), Cremei (Hubka et al. 2016b), Aspergillus (Chen et al. 2017), Fumigati (Hubka et al. 2017), Candidi (Hubka et al. 2018a) and two other newly described sections (Visagie et al. 2017).

Aspergillus section Restricti comprises xerophilic species that are able to grow on substrates with low water activity and in extreme environments. More than 300 strains were characterized and taxonomically revised. The monographic study recognised 21 species, including 14 new ones. In this study, several advanced tools for species delamination were firstly tested on fungal model (Sklenář et al. 2017).

Aflatoxins and ochratoxins, produced by the members of Aspergillus section Flavi, are among the most important mycotoxins. Using a polyphasic approach combining phenotype, physiology, sequence and extrolyte data, we described eight new species (33 spp. in total). Only six species in the section did not produce any known mycotoxins (Frisvad et al. 2019). The importance of this comprehensive study is demonstrated by its immediate citation feedback (google scholar citations: 74, Feb 2020).

The progressive direction of our research represents the work on species delamination methods and study of speciation mechanisms. Species of the section Fumigati are important human pathogens responsible for the 600,000 deaths annually. We presented new tools for the species delimitation and revised number of species and newly defined species limits in this group. We also showed the ability of interspecies mating what opens a possibility for the origin of new species with the new antifungal susceptibility patterns. We also compared currently used methods for antifungal susceptibility testing, demonstrated their limitations and recommended new approaches for the determination of therapeutic dozes (Hubka et al. 2018, Lysková et al. 2018).

Selected outputs 2015-2019. Members of Lab 112 were corresponding or/and first authors (Hubka et al. 2015, 2016a, 2016b, 2017, 2018a, 2018b, Sklenář et al. 2017, Lysková et al. 2018). For the study of Jurjević et al. (2016) we provided some strains, species descriptions, we generated most of the molecular data and we performed all phylogenetic analyses. For the studies of Chen et al. (2017) and Visagie et al. (2017) we provided strains together with related molecular data. For the study of Frisvad et al. (2019) we contributed by the description (incl. strains, and sequence data) of several species and by providing strains and sequence data for other spp. (total contribution cca 30%).

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2) **Hubka V, Nováková A**, Peterson SW, Frisvad JC**, Sklenář F**, Matsuzawa T, Kubátová A, **Kolařík M** (2016a) A reappraisal of Aspergillus section Nidulantes with descriptions of two new sterigmatocystin-producing species. Plant Syst Evol 302:1267-1299
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6) **Hubka V, Dudová Z**, Kubátová A, Frisvad JC, Yaguchi T, Horie Y, Jurjević Ž, Hong S-B, **Kolařík M** (2017) Taxonomic novelties in *Aspergillus* section Fumigati: *A. tasmanicus* sp. nov., induction of sexual state in *A. turcosus* and overview of related species. Plant Syst Evol 303:787-806
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2) Visagie CM, Yilmaz N, Renaud JB, Sumarah MW, **Hubka V**, Frisvad JC, Chen AJ, Meijer M, Seifert KA, (2017) A survey of xerophilic *Aspergillus* from indoor environment, including descriptions of two new section Aspergillus species producing eurotium-like sexual states. MycoKeys 19: 1.
3)
4) Lyskova P, **Hubka V**, Svobodova L, Barrs V, Dhand NK, Yaguchi T, Matsuzawa T, Horie Y, **Kolarik M**, Dobias R (2018) Antifungal susceptibility of the *Aspergillus viridinutans* complex: comparison of two in vitro methods. Antimicrob Agents Chemother: AAC. 01927-01917
5) **Hubka V, Nováková A**, Jurjević Ž, Sklenář F, Frisvad JC, Houbraken J, Arendrup MC, Jørgensen KM, Siqueira JP, Gené J., **M. Kolařík** (2018a) Polyphasic data support the splitting of *Aspergillus candidus* into two species; proposal of *Aspergillus dobrogensis* sp. nov. Int J Syst Evol Microbiol 68: 995-1011
6) Hubka V, Barrs V, Dudová Z, Sklenář F, Kubátová A, Matsuzawa T, Yaguchi T, Horie Y, Nováková A, Frisvad Talbot JJ, Kolařík M (2018b) Unravelling species boundaries in the Aspergillus viridinutans complex (section Fumigati): opportunistic human and animal pathogens capable of interspecific hybridization. Persoonia 41: 142-174.
7) Frisvad, J. C., V. Hubka, C. N. Ezekiel, S. B. Hong, A. Nováková, A. J. Chen, M. Arzanlou, T. O. Larsen, F. Sklenář, W. Mahakarnchanakul, R. A. Samson and J. Houbraken (2019). "Taxonomy of Aspergillus section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins." Studies in Mycology 93: 1-63.

Clinical fungi

Fungi causing mycoses are important pathogens of humans and animals. We focused on the epidemiology, taxonomy, clinical relevance of dermatophytes and non-dermatophytic moulds. 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 (2012-2020). The preliminary results were already published in the local and international papers (4 papers, not shown). Our outputs cover case reports (Chrenková et al. 2015, Hubka et al. 2015, Lyskova et al. 2015, Tyll et al. 2016, Řehulka et al. 2017, Lyskova et al. 2017), taxonomic studies (Hubka et al. 2015, Lyskova et al. 2015). We also firstly evaluated the potential of the biocontrol agent, *Pythium oligandrum* in the dermatomycosis treatment (Gabrielová et al. 2018).

Selected outputs 2015-2019. Selected out of 22 publications. Team members were first or/and corresponding authors in case of Hubka et al. (2015), Řehulka et al. (2017) and Gabrielová et al. (2018). We contributed by the identification of the strains, phylogenetic analyses and participation on the paper writing in case of Tyll et al. (2016), Chrenková et al. (2015) and Lyskova et al. (2015, 2017).

1) Chrenkova V, **Kolarik M**, Hubacek P, Kolarik J, Simonek J, Lischke R, Drevinek P (2015) Possible *Tyromyces fissilis* (Basidiomycota, Polyporales) co-infection in a lung transplant recipient Folia Microbiologica 60: 33-5.
2) **Hubka V**, Nissen CV, Jensen RH, Arendrup MC, **Cmokova A**, Kubatova A, Skorepova M, Kolarik M (2015) Discovery of a sexual stage in *Trichophyton*

onychocola, a presumed geophilic dermatophyte isolated from toenails of patients with a history of T. rubrum onychomycosis. Medical Mycology. 53: 798-809.
3) Lyskova P, **Hubka V**, Petricakova A, Dobias R, **Cmokova A, Kolarik M** (2015) Equine dermatophytosis due to *Trichophyton bullosum*, a poorly known zoophilic dermatophyte masquerading as *T. verrucosum*. Mycopathologia. 180:407-419.
4) Tyll T, Lyskova P**, Hubka V**, Muller M, Zelenka L, Curdova M, Tuckova I**, Kolarik M,** Hamal P (2016) Early diagnosis of cutaneous mucormycosis due to *Lichtheimia corymbifera* after a traffic accident. Mycopathologia. 181: 119-124.
5) Řehulka J., Kolařík M**., Hubka V.** (2017) Disseminated infection due to *Exophiala pisciphila* in Cardinal tetra, Paracheirodon axelrodi,
6) Lyskova P, Kubanek M, **Hubka V**, Sticova E, Voska L, Kautznerova D, **Kolarik M**, Hamal P, Vasakova M (2017) Successful posaconazole therapy of disseminated alternariosis due to *Alternaria infectoria* in a heart transplant recipient. Mycopathologia:1-7. DOI: 10.1007/s11046-016-0094-4
7) **Gabrielová A**, Mencl K, Suchánek M, Klimeš R**, Hubka V, Kolařík M** (2018) The oomycete *Pythium oligandrum* can suppress and kill the causative agents of dermatophytoses. Mycopathologia 183: 751-764.

Naming new fungi is a crucial step accelerating further research. We collaborated on the description of six new genera and tens of new species of acidophilic, endophytic, soil and cave fungi and mushrooms growing in the natural European forests. This collaborations resulted in the description of species important for the wild life conservation and biotechnology. Naming the fungi is always the way how to attract the attention to the particular fungal group and promote its further research. This activity, represented by 25 scientific publications (2015-2019), can be demonstrated on following projects.

Our team studied biologically active secondary metabolites of the genus *Biatriospora* (Stodůlková et al. 2015) which produced five new antibiotics. The producer strain couldn't be ascribed to any known species and we conducted its taxonomic revision, in order to find a proper name (Kolařík et al. 2017). Our work expanded the *Biatriospora* taxonomic and ecological concept by describing four new species found as endophytes of woody plants. The new name *B. antibiotica* was chosen for the producer strain, to attract attention to our results. Our findings show that this genus is more diverse and has more host associations than has been described previously. The possible adaptations enabling the broad ecological range of these fungi are discussed.

Highly acidic soils harbour large proportion of undescribed fungal diversity (Hujslová et al. 2017). Some of our strains belonged into the proximity of *Penicillium oxalicum* which is one of the most frequently patented fungal species, with huge biotechnological potential. Thus we decided to describe our strain as a two new species (Kubátová et al. 2019) using complex data about genotype and phenotype (incl. secondary metabolite production). This study was the first, after many decades, which expanded the diversity of species from *P. oxalicum* species group.

In the previous study (Vohník et al. 2010, PLoS ONE 7(6): e39524) the novel type of mycorrhizal symbiosis was discovered. The identity of the fungal symbiont was mysterious. In our recent study we identified the fungus as a basidiomycete *Kurtia argillacea* (Kolařík et Vohník 2018). This study showed, that rDNA sequences, the widely used DNA barcode, is not reliable for the identification or phylogenetic

placement of this fungus. This study brings attention to the widely ignored sources of artefacts in taxonomic and phylogenetic studies.

Practical wildlife protection rely upon so called umbrella species which are the actual subjects of protection. Such red-listed species are rare, but visually remarkable (i.e. macromycetes) indicators of natural habitats. Our laboratory contributed by the molecular taxonomy and phylogeny of rare fungi such as *Chromosera cyanophylla, Galerina* spp., *Gymnopilus suberis* and *Clitocybula* spp. and others (Holec et al. 2015, Holec et al. 2016, Holec et al. 2017, Antonín et al. 2019). Among the new species, *Tricholomopsis badinensis*, an indicator or old-grown species, was described (Holec et al. 2019).

Selected outputs 2015-2019. Selected from 25 scientific publications. Members of Lab 112 were corresponding or/and first authors (Kolařík et al. 2017, Kolařík et Vohník 2018, Kubátová et al. 2109) or contributed by providing molecular taxonomic and phylogenetic analyses.

1) Holec J, Kříž M, Beran M, **Kolařík M** (2015) *Chromosera cyanophylla* (Basidiomycota, Agaricales) - a rare fungus of Central European old-growth forests and its habitat preferences in Europe. Nova Hedwigia 100: 189-204.
2) Holec J, Kríž M, **Kolařík M,** Žák M (2016) Mediterranean fungus *Gymnopilus suberis* discovered in Central Europe–a consequence of global warming? Sydowia 68:69
3) **Kolařík M**, Spakowicz DJ, Gazis R, Shaw J, Kubátová A, **Nováková A**, **Chudíčková M**, Forcina GC, Kang KW, Kelnarová I, Skaltsas D, Portero CE, Strobel SA, Narváez-Trujillo A (2017) *Biatriospora* (Ascomycota: Pleosporales) is an ecologically diverse genus including facultative marine fungi and endophytes with biotechnological potential. Plant Syst Evol 303 (1): 35–50.
4) Hujslová M, Kubátová A, Bukovská P, **Chudíčková M, Kolařík M** (2017) Extremely acidic soils are dominated by species-poor and highly specific fungal communities. Microb Ecol: 321–337.
5) Holec J, Vašutová M, **Kolařík M**, Kříž M (2017) *Galerina saxicola* (Fungi, Agaricales) is conspecific with *G. stordalii* and new data on ecology of the latter species. Plant Syst Evol 303:23-33
6) **Kolařík M,** Vohník M (2018) When the ribosomal DNA does not tell the truth: The case of the taxonomic position of Kurtia argillacea, an ericoid mycorrhizal fungus residing among Hymenochaetales. Fungal Biol 122:1-18
7) Kubátová A, Hujslová M, Frisvad JC, **Chudíčková M, Kolařík M** (2019) Taxonomic revision of the biotechnologically important species *Penicillium oxalicum* with the description of two new species from acidic and saline soils. Mycological Progress: 18, 215-228.
8) Holec J, Kunca V, **Kolařík M** (2019) *Tricholomopsis badinensis* sp. nov. and *T. sulphureoides* - two rare fungi of European old-growth forests. Mycological Progress 18: 321-334.
9) Antonín V, Borovička J, Holec J, Piltaver A, **Kolařík M** (2019). Taxonomic update of *Clitocybula* sensu lato with a new generic classification. Fungal Biology 123: 431-447.

Research activity and characterization of the main scientific results

The Laboratory operates since 2011 as a 'confederation of PIs', where aside of the Head, other 4 senior members direct their sub-teams and regularly apply for funding (Drs. Osička, Bumba, Mašín and Kamanová) obtaining grants from the CSF and Ministries of Education, Health or Agriculture.

Still steered and coordinated by the Head of the Laboratory, this structure gives the senior scientists (PIs) a high degree of freedom in conducting fairly independent research, while still allowing to maintain the necessary cohesion of the coordinated research efforts.

Due to own funding, while remaining members of a single integrated and intrinsically cooperative research team, the senior members of the team are leading their subgroups in the Laboratory, being able to develop their own independent research ideas and projects. These typically exhibit a good level of synergy with the research developed by the other research-leading team members, while sharing the research infrastructure, methodical approaches and know-how represents a particular asset. The individual subgroup projects are typically highly related and mutually cross-fertilizing, focusing mainly on the central theme of the structure-function relationships underlying the mechanism of action of the adenylate cyclase toxin and its mechanisms of subversive activity on host immune cell functions. However, additional topics are developed as well and as a whole, the 'confederation of PIs' still operates in a concerted manner as one large integrated research team that exhibits a high level of internal communication, collaboration and of technical and intellectual synergy between its senior members and involved students and postdocs. 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 maintain a high level of awareness on who is doing what and by which methods. This cross-fertilizes research collaborations inside of our fairly large team.

The spectrum of methods and approaches is broad and comprises all necessary technologies, including structural biology (X-ray crystallography, protein NMR structure solving, SAXS, CD and fluorescence spectroscopy), conventional protein biochemistry, reverse bacterial genetics, cell biology approaches, various omics and the use of animal models of infection, including targeted transgenic mice design and use. This research environment offers a unique opportunity to our trainees to get a broad and complete scientific culture and acquire a number of technical skills useful for all kinds of biomedical research careers.

<u>Over the evaluated period of the years 2015-2019 the team was able to obtain:</u>

8 regular CSF grants

1 ExPro CSF Excellence grant (2019 – P. Šebo)

1 Ministry of Health grant

1 Ministry of Agriculture grant

- ***participated in 1 H2020 IMI-2 and 1 H2020 EU grant***
- ***and 2 OP VVV infrastructure projects (EATRIS)***.

As a result, the operation of the lab was very well funded and the team was highly productive, publishing 19 core papers and authoring or co-authoring 34

additional papers published over the evaluated period in recognized international journals.

In parallel to basic research, the team worked on 2 Contract Research projects with industrial partners and applied for 3 EU patents. To save costs, the patent applications were, however, retracted once the Evaluation and Option agreements with a major pharmaceutical company expired without progressing towards a license deal.

One contractual applied research project was funded by a private investor and yielded the development of a next generation whole cell pertussis vaccine that is currently under evaluation in immunized primates at CBER FDA USA with support of the NIAID NIH of the USA. There is a good prospect of yielding a license deal with a major global vaccine manufacturer in the event of positive outcome of the preclinical efficacy study in primates.

The research activities of the team over the evaluated period (2015-2019) can be roughly broken down into the following 6 subtopics (ST):

ST1 Structure, function and molecular mechanism of action of adenylate cyclase toxin (ACT):

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 phagocytes towards ablation of their bactericidal capacities (Dr. Šebo)*
4) *Role of ACT permeabilization of the epithelial barrier (Drs. Šebo + Osička)*
5) *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 T3SS function:

1) *Expression and biological role of the T3SS system of B. pertussis (Dr. Kamanová)*
2) *Structure, function and immunosuppressive activity of B. pertussis FHA (Dr. Šebo and Bumba)*

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)*
2) *Role of the RtxA cytolysin in virulence and invasiveness of Kingella kingae (Dr. Osička)*

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

ST5 Role of the self-aggregation motif of ameloblastin in formation of enamel (Dr. Osička)

ST6 Contract research on development of novel pertussis vaccines (confidential)

SUMMARY OF THE MOST IMPORTANT RESULTS AND ACHIEVEMENTS IN 5 YEARS.

The main scientific results achieved within the evaluation period can be stratified according to the major developed lines of research, highlighted below, by using selected examples of the most important research achievements of our team:

ST1 Structure, function and molecular mechanisms of action of the adenylate cyclase toxin (ACT):

1) ***We have determined the site of adenylate cyclase toxin (ACT) binding on its receptor, the $\alpha_M\beta_2$ integrin CD11b/CD18, known as the complement receptor 3 (CR3)*****,** or Mac-1, which is expressed on myeloid phagocytic cells. In the report by **Hasan *et al.* (2015)** we showed that the initial interaction of ACT with N-linked glycans involves a multivalent low-affinity interaction that precedes a specific interaction of a segment of the RTX domain of ACT with a particular positively charged loop in the thigh domain of the CD11b subunit of the integrin, as we show in the report by **Osicka *et al.* (2015)**. In this seminal work we used a set of a dozen of stably transfected and expression level-calibrated CHO cell transfectants expressing chimeric integrins consisting of the CD18 subunit paired with a series of CD11b/CD11c hybrid subunits with swapped domains. This allowed to pin down the ACT binding site on CD11b and show that ACT preferentially binds the closed conformation of the integrin. Moreover, **we showed that ACT is a unique ligand of CR3** in that it does not bind through its I domain, where other biological ligands bind, thus triggering outside-in signaling of the integrin. In contrast, **the subversive binding of ACT to the thigh domain of the closed integrin molecule allows to bypass the integrin signaling activation.** We have then further demonstrated that delivery of the AC enzyme domain of ACT into cytosol of phagocytes and elevation of cellular cAMP level by its calmodulin-activated action does prevent the activation of CR3 signaling following engagement with is iC3b ligand-opsonized particles. We showed that **production of cAMP by ACT causes inactivation of the central receptor-associated transmitter tyrosine kinase Syk and thereby blocks integrin signaling and initiation of opsonophagocytic uptake of complement opsonized particles by the phagocytes.** By this demonstration we have **provided a mechanistic explanation of the dramatic efficacy of ACT action in suppressing the opsonophagoctic bactericidal activity of host phagocytes**. This works explains the major role played by the ACT in immune evasion of the pathogenic *Bordetellae*.

Osicka* R., *et al.* (2015). *Bordetella* adenylate cyclase toxin is a unique ligand of the integrin complement receptor 3. **eLife 2015;10.7554/eLife.10766;**
Hasan S, *et al.* (2015). Interaction of Bordetella adenylate cyclase toxin with complement receptor 3 involves multivalent glycan binding. ***FEBS Lett***. **589**:374-9.

2) ***We have thoroughly analyzed the interaction of ACT with target cell membrane*****,** showing that the transmembrane segments of its complement receptor do not participate in toxin action (**Wald *et al.* 2015**). Quite important was the discovery that the 'AC-to-Hly linking segment' of CyaA participates in membrane insertion of the toxin and that the balance between its pore-forming and AC translocating activity is maintained by the clusters of the negatively charged residues in this segment (**Masin *et al.* 2016**). Another important discovery came

from identification of the Y940 residue as being crucial for the initial interaction and penetration of the toxin molecule into lipid bilayer of the target cell membrane (**Masin *et al.* 2017**). Last not least we have characterized involved structures and defined the role of individual residues located in an amphipathic segment in membrane penetration and translocation of the toxin (**Roderova *et al.* 2019**)

Roderová J.*et al.* (2019). Residues 529 to 549 participate in membrane penetration and pore-forming activity of the *Bordetella* adenylate cyclase toxin. ***Sci Rep.* 9(1):5758**.
Mašín, J. *et al.* (2017). The conserved tyrosine residue 940 plays a key structural role in membrane interaction of Bordetella adenylate cyclase toxin. ***Sci. Rep.* 7(1):9330.**
Mašín J.* *et al.* (2016). Negatively charged residues of the segment linking the enzyme and cytolysin moieties restrict the membrane-permeabilizing capacity of adenylate cyclase toxin. ***Sci. Rep.* 6:29137**
Wald T. *et al.* (2016). Transmembrane segments of complement receptor 3 do not participate in cytotoxic activities but determine receptor structure required for action of Bordetella adenylate cyclase toxin. ***Pathog. Dis.* 74(3). pii: ftw008.**

3) ***A major field of our research activity consisted in the analysis of the biological effects resulting from ACT action on CR3-expressing myeloid cells***, in particular the synergy of its cAMP-elevating and pore-forming activities and the interference of such signaling with the bactericidal functions of the sentinel cells of the immune defense. We found that the pore-forming activity of ACT triggers MAPK activation due to potassium efflux and this can trigger dendritic cell maturation (**Svedova *et al.* 2016**). In a subsequent study we were the first ones to be able to address the biological role of the pore-forming activity of ACT in *Bordetella* virulence, due to constructing a non-hemolytic toxin that still suppressed phagocyte action by cAMP signaling (**Skopova *et al.* 2017**). We showed that the pore-forming activity synergizes with cAMP signaling and provokes attraction of inflammatory cells into infected tissue, accounting to large part for the virulence of *B. pertussis* infection of the lungs. A seminal discovery of our team was that by an as yet unknown mechanism **the signaling of toxin-produced cAMP through PKA triggers activation of the tyrosine phosphatase SHP-1** that plays a key role in leukocyte biology and receptor signaling. We showed that this blocks TLR signaling-mediated activation of AP1 and induction of iNOS, thereby preventing bactericidal NO production in macrophages and promoting survival of internalized *Bordetella* bacteria (**Cerny *et al.* 2015**). We further deciphered the pathways by which ACT action instantaneously blocks NDPH oxidase assembly and oxidative burst of primary human neutrophils, promoting bacterial evasion to sentinel cells of the immune system through SHP-1 activation, ERK inhibition and Epac-mediated Inhibition of phospholipase C (**Cerny *et al.* 2017**). Moreover, we showed that through inhibition of ERK signaling and SHP-1 activation **as low as 50 pM ACT provokes stabilization of BimEL and activation of Bax, thereby committing human macrophage cells to apoptosis** (**Ahmad *et al.* 2016**). Most recently we discovered that even lower concentrations of ACT (22 pM) can hijack cAMP signaling of primary human monocytes, block their differentiation into more bactericidal macrophages. Furthermore, for the first time we reported that **cAMP signaling elicited by ACT can trigger de-differentiation of primary human alveolar macrophages back to monocyte like cells** (**Ahmad *et al.* 2019**). This represents a fundamental discovery on how bacterial toxins can manipulate host defense and promote immune evasion of a pathogen, as tissue macrophages are considered to be terminally differentiated and self-renewable. Our unbiased phosphoproteomic analysis of cAMP signaling in dendritic cells uncovered the

pathways leading to immunosubversive action of the toxin and immunosuppressive IL-10 secretion (**Novak *et al.* 2017**).

4) Finally, in collaboration with a team form Iceland ***we discovered that cAMP elevation in polarized airway epithelial cells disrupts tight junctions and compromises the burrier function of airway epithelia*** (**Hasan *et al.* 2018**). Pursuing on this observation now represents a major field of our research with the aim of explaining the molecular causes of the catarrhal phase of pertussis and transmission of the pathogen from host to host (**ExPro project**).

Ahmad JN, *et al.* (2019). *Bordetella* Adenylate Cyclase Toxin Inhibits Monocyte-to-Macrophage Transition and Dedifferentiates Human Alveolar Macrophages into Monocyte-like Cells. ***mBio* 10(5): e01743-19**.

Hasan S., Ur Rahman W., **Sebo P.** and R. Osicka*. (2019). Distinct Spatiotemporal Distribution of Bacterial Toxin-Produced Cellular cAMP Dierentially Inhibits Opsonophagocytic Signaling. ***Toxins* 11, 362**

Hasan, S.*et al.* (2018). *Bordetella pertussis* adenylate cyclase toxin disrupts functional integrity of bronchial epithelial layers. ***Infect. Immun. 86:e00445-17***,

Novák, J. et al. (2017). Phospho-proteomics of cAMP signaling of Bordetella adenylate cyclase toxin in mouse dendritic cells. ***Sci. Rep.* 7:** 16298. DOI:10.1038/s41598-017-14501-x

Škopová, K. *et al.* (2017). cAMP-elevating capacity of the adenylate cyclase toxin-hemolysin is sufficient for lung infection but not for full virulence of *Bordetella pertussis.* ***Infect. Immun*. 85 (6**) e00937-16. doi: 10.1128/IAI.00937-16.

Černý. O. *et al.* (2017). cAMP Signaling of Adenylate Cyclase Toxin Blocks the Oxidative Burst of Neutrophils through Epac-Mediated Inhibition of Phospholipase C Activity. ***J. Immunol*. 198:** 1285-1296. DOI: 10.4049/jimmunol.1601309

Švédová, M.*et al.* (2016). Pore-formation by adenylate cyclase toxoid activates dendritic cells to prime CD8+ and CD4+ T cells. ***Immunol. Cell. Biol.* 94:**322-333

Ahmad, JN *et al.* (2016). cAMP signaling of *Bordetella* adenylate cyclase toxin through the SHP-1 phosphatase activates the BimEL-Bax pro-apoptotic cascade in phagocytes. ***Cell. Microbiol***. **18**: 384–398

Černý, O *et al.* (2015). *Bordetella pertussis* Adenylate Cyclase Toxin Blocks Induction of Bactericidal Nitric Oxide in Macrophages through cAMP-dependent activation of the SHP-1 Phosphatase. ***J. Immunol.* 194**: 4901–4913.

5) ***We have achieved a major breakthrough in the understanding of the molecular mechanism of T1SS-mediated secretion of RTX proteins as a whole and of the calcium-triggered folding of ACT in particular.*** We solved by X-ray crystallography the 3D structure of the last RTX block V of ACT and defined its C-terminal folding scaffold, the formation of which through a cation-π-π interaction enables formation of a loop that binds the first calcium ion and triggers vectorial C- to N-terminal calcium loading-dependent stacking of β sheets into β-rolls, promoting folding of the entire 700-residue long RTX domain (as followed by HSQC NMR and SAXS) into 5 β-rolls forming the CR3 receptor binding domain of ACT (**Bumba *et al.* 2015**). This work sets a new paradigm, showing that calcium-dependent folding of RTX proteins excreted across bacterial envelopes through the T1SS apparatus occurs co-secretionally and yields formation of Brownian molecular ratchets that prevent backsliding of the translocated polypeptide chain, thereby providing a kinetic contribution and accelerating the process of the ATP-ase driven secretion of the polypeptide (**Bumba *et al.* 2015**).

Bumba L. *et al.* (2016). Calcium-driven folding of RTX domain β-rolls ratchets translocation of RTX proteins through Type I secretion ducts. ***Mol. Cell* 62:**47-62.

ST2 Other virulence mechanisms of *B. pertussis* and T3SS function:

1) ***The sub-team led by Dr. Kamanová initiated e new line of research in our 'PI confederation' lab on the biological activity and role in virulence of the T3SS effector BteA,*** the biochemical basis of cytotoxicity of which remains unknown. The sub-team discovered that an Ala residue (A503) in BteA of *B. pertussis* that renders it significantly less cytotoxic than is the BteA of *B. bronchiseptica.* The results revealed that this represents an evolutionary adaptation related to switch from a chronic infection phenotype in mammals of *B. bronchiseptica* towards an acutely virulent infection by *B. pertussis,* where the reduced activity of BteA enables higher inflammation and likely more efficient transmission (**Bayam *et al.* submitted**)

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

1) Under this subtopic the sub-team led by Dr. Bumba with external collaborators solved the X-ray structure of the protein FrpD that binds the N-terminus of FrpC of *N. meningitidis* and demonstrated that this interaction allows a very tight covalent bond-mediated adhesion of the bacterium on host cell surface.

Sviridova E *et al.* Structural basis of the interaction between the putative adhesion-involved and iron-regulated FrpD and FrpC proteins of *Neisseria meningitidis*. **Sci Rep. 13;7:40408.**

2) The team lead by Dr. Bumba with external collaborators solved by NMR the solution structure of the unique self-processing module of large RTX proteins discovered in our laboratory in 2004. The present work revealed a unique fold and unprecedented mechanism of an Asp-Pro bond cleavage that plays a role in biological activity of the ApxIVA protein of the porcine pathogen *Actinobacillus pleuropneumoniae* and contributes to its virulence

Kubáň *et al* (2020) Structural basis of Ca2+-dependent self-processing activity of Repeat in Toxin (RTX) proteins. ***mBio* in press**

3) The sub-team of Dr. Osička analyzed the cytotoxic activity of recombinant RtxA cytolysin of the emerging pediatric pathogen *Kingella kingae* and demonstrated the role of its acylation in biological activity.

Osickova A *et al.*(2018) Cytotoxic activity of *Kingella kingae* RtxA toxin depends on post-translational acylation of lysine residues and cholesterol binding. ***Emerg Microbes Infect.* 7(1):178**

ST5 Role of a self-aggregation motif of ameloblastin in enamel formation

The sub-team led by Dr. Osička developed a protein structure-oriented project that lead to the discovery of a novel Y/F-x-x-Y/L/F-x-Y/F protein self-assembly motif that is evolutionarily conserved from the first tetrapods to man. In collaboration with experts on enamel formation they further showed that the motif is crucial for

higher order structure self-assembly of the key intrinsically disordered enamel matrix proteins ameloblastin and amelogenin and by using targeted mutations in mice demonstrated that it accounts for hydroxyapatite crystallite formation and arrangement that confers the hardness on enamel of teeths, the hardest biological

tissue that enabled food processing and its constitution played a major role in evolution of all tetrapods.

Wald T *et al.*(2017). Intrinsically disordered proteins drive enamel formation via an evolutionarily conserved self-assembly motif. ***Proc Natl Acad Sci USA*** **114(9):E1641-E1650**

ST6 Contract research on development of novel pertussis vaccines

Under this contractual and confidential research collaboration with a private investor and with a major pharmaceutical vaccine manufacturing company we have developed novel ACT based antigens for improvement of acellular pertussis vaccine composition and a next generation whole cell pertussis vaccine with reduced reactogenicity. The latter was produced in cGMP quality and formulated into an experimental pediatric pentavaccine by a contractual partner and is currently under evaluation for efficacy and reactogenicity in the immunized non-human primates, olive baboons, at the CBER FDA USA with financial support of the NIAID NIH of the USA. Providing positive outcome of the tests, the prospect of licensing the vaccine to a major global vaccine manufacturer will be realistic.

Research activity and characterisation of the main scientific results

In the evaluation period 2015-2019 the Laboratory of Biotransformation earned following grants:
Czech Science Foundation: 7 standard grants, 2 junior grants, 1 bilateral grant (together with DFG)
Czech Ministry of Education: 17 INTER-EXCELLENCE grants, 2 projects CONTACT (with US partner)
Czech Ministry of Health: 1 grant
Czech Technology Agency: 1 grant

Minor and neglected flavonolignans and metabolites, and inter-individual variability in the response to flavonolignan consumption. Minor flavonolignans such as 2,3-dehydrosilybin, 2,3-dehydrosilychristin and 2,3-dehydrosilydianin, anhydrosilychristin, and 2,3-dehydroanhydrosilychristin were isolated from *Silybum marianum* and/or synthesized [1-4]. Moreover, the importance of these compounds in silymarin has been summarized [5] and new HPLC method was developed [6]. In cooperation with our partners, we performed studies of the biotransformation of these compounds by human fecal bacteria, as well human liver microsomes and hepatocytes [7-9]. Simultaneously, we enzymatically prepared authentic standards of potential metabolites and validated the methods for their identification [10-12]). Purified (potential) metabolites were tested (see below) and were used for biological studies, including antioxidant tests, screening in the CZ-OpenScreen platform, and testing in cooperation with the Palacký University Olomouc, Charles Univ. in Prague and others. The standards were also provided to the partners in the COST Action FA1403 POSITIVe in order to develop and validate methods determining their bioavailability [13].
[1] Pyszková, Biler, Biedermann et al., *Free Radic. Biol. Med.* **90**, 114, 2016; IF 5.606, 48 cit. [2] Biedermann et al., *J. Nat. Prod.*, **79**, 3086, 2016; IF 3.281, 14 cit. [3] Biedermann, D. et al., *Phytochem. Lett.,* **30**, 14, 2019; IF 1.338, 2 cit. [4] Valentová et al. *Proceedings,* **11**, 21, 2019, 2 cit. [5] Chambers et al., *Food Res. Int.*, 2017, **100**, 339-353; IF 3.520, 24 cit. [6] Petrásková et al., *Foods,* **9**, 116, 2020; IF 3.011. [7] Vrba, … Křen, et al., *J. Pharm. Biomed. Anal.*, **152**, 94, 2018; IF 2.983, 5 cit. [8] Vrba, … Křen, et al., *J. Pharm. Biomed. Anal.*, **178**, 112972, 2020; IF 2.983. [9] Valentová et al., *Metabolites,* **10**, 29, 2020; IF 3.303. [10] Valentová et al., *Int. J. Mol. Sci.,* **18**, 2231, 2017; IF 3.687, 5 cit. [11] Valentová et al., *Int. J. Mol. Sci.,* **19**, 2349, 2018; IF 4.183, 4 cit. [12] Begines, Biedermann et al., *J. Agric. Food Chem.,* **67**, 7281, 2019; IF 3.571. [13] Koistinen, … Valentová, et al., *Metabolites,* **8**, 46, 2019; IF 3.303, 2 cit.

Biophysical and biological activity of flavonoids and their metabolites. Various biological activities of the compounds isolated or synthesized in our laboratory were researched in cooperation with our partners: Optical and acido-basic properties of quercetin and its analogues [1] and the potential of flavonolignans as a novel class of sodium pump inhibitors [2] were studied in cooperation with the Faculty of Science, Palacký University, Olomouc. Chelation of flavonoids with iron or copper [3-4] and the effect of the flavonolignans and their sulfated conjugates on platelet aggregation and blood vessels [5] were investigated in the frame of our joint project with the Faculty of Pharmacy, Charles University in Hradec Králové. The effect on bilirubin concentrations in mice [6] is the main finding stemming from our collaboration with the 1st Faculty of Medicine of the Charles University in Prague. Antioxidant, anti-

inflammatory, and multidrug resistance modulation activity of silychristin derivatives [7] were studied in cooperation with the University of Chemistry and Technology in Prague. 2,3-Dehydrosilybin A/B was found to act as a pro-longevity and anti-aggregation compound [8] and both silybin and its 2,3-dehydro-derivative inhibited basal cell carcinoma growth via suppression of mitogenic signaling and transcription factors activation [9]. High *in vitro* activity of flavonolignans against *Leishmania infantum* and *L. donovani* was uncovered for 2,3-dehydroisosilybin A and 2,3-dehydrosilybin A and B [10]. The collaboration with the Faculty of Medicine and Dentistry, Palacký University in Olomouc focused in this period on the redox properties of individual moieties in quercetin structure [11], the effect of flavonolignans on Nrf2 activation and regulation of related genes [12] and on the dermal delivery and skin protective activity of silymarin and its flavonolignans [13-14]
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[1] Biler, Biedermann, et al., *Phys. Chem. Chem. Phys.* **19**, 26870, 2017; IF 3.906, 6 cit. [2] Kubala, … and Biedermann, *Front. Physiol*. **7**, 115, 2016; IF 3.882, 8 cit. [3] Catapano, … Valentová, et al., *Nutrients* **9**, 1193, 2017; IF 4.196, 1 cit. [4] Tvrdý, … and Valentová, *J. Inorg. Biochem.* **189**, 115, 2018; IF 3.224, 1 cit. [5] Pourová, … Biedermann, et al., *Nutrients* **11**, 2286, 2019; IF 4.171. [6] Šuk, … Biedermann, et al., *Oxid. Med. Cell. Longev.* 2019, Article ID 6026902, 12 p.; IF 4.868, 4 cit. [7] Viktorová, … Biedermann, et al., *Antioxidants,* **8**, 303, 2019; IF 4.520. [8] Filippopoulou, … Biedermann, et al., *Free Radical Bio. Med.,* **103**, 256-267, 2017; IF 5.606, 11 cit. [9] Tilley, … Biedermann, et al., *Mol. Carcinogen.,* **55**, 3-14; IF 4.185; 14 cit. [10] Olías-Molero, … Biedermann, et al. *Molecules,* **23**, 1560, 2018; IF 3.098, 1 cit. [11] Heřmánková et al, *Free Radical Bio. Med.* **143**, 240, 2019; IF 5.657, 1 cit. [12] Roubalová, … Křen, et al., *Fitoterapia* **119**, 115, 2017; IF 2.698, 11 cit. [13] Kosina, … Biedermann, et al., *Molecules* **24**, 61, 2019; IF 3.060, 5 cit. [14] Vostálová, … Biedermann, et al., *Molecules* **24**, 1022, 2019; IF 3.060, 5 cit.

Hybrid molecules and other derivatives. Various hybrid molecules aimed at improving the bioactivities of flavonoids and studying redox interactions were synthesized in the laboratory and their properties investigated. Isoquercitrin esters with mono- or dicarboxylic or aromatic acids and their homologues [1-2] were efficient inhibitors of lipid peroxidation with increased lipophilicity. Selective galloylation at C7-OH increased the effect of 2,3-dehydrosilybin on human umbilical vein endothelial cells [3-5]. We also prepared retinoyl-flavonolignan hybrids with improved antioxidant properties [6]. Finally, a series of antioxidants was designed and synthesized based on conjugation of silybin with L-ascorbic acid, trolox alcohol or tyrosol *via* a C-12 aliphatic linker. The silybin-ascorbic acid conjugate exhibited excellent electron donating ability and displayed the best activities (IC_{50} = 30 μM) in terms of inhibition lipid peroxidation [7].

[1] Vavříková et al., *Int. J. Mol. Sci.* **17**, 899, 2016; IF 3.226, 11 cit. [2] Heřmánková-Vavříková et al., *Int. J. Mol. Sci.* **18**, 1074; IF 3.687, 8 cit. [3] Karas et al., *J. Nat. Prod.* **79**, 812, 2016; IF 3.281, 7 cit. [4] Pivodová et al. *Pharmazie,* **71**, 478-483, 2016; IF 1.126, 1 cit. [5] Karas et al. *Food Chem. Toxicol.* **105**, 223-240, 2017; IF 3.977, 15 cit. [6] Chambers et al., *Antioxidants,* **8**, 236, XXX

Nitrilases of bacterial, fungal and plant origin hydrolyze nitriles at ambient temperature and near neutral pH. This is usually impossible with chemical tools as the nitrile bond is very stable. Therefore, nitrilases are attractive for synthetic organic chemistry, where their enantio- and regioselectivity is also appreciated. With the increasing amount of sequential data, the strategy based on database searches gains increasing importance.

We have searched the genomes of filamentous fungi, whose nitrilases were almost unexplored. Fifteen new nitrilases were reported by us, among them the first nitrilases in *Basidiomycota* fungi. This project focused on the enzymes´ sequence

analysis, structure-activity relationships, taxonomical distribution and applications in the production of fine chemicals and in eco-technologies.
The main results were e.g. the characterization of new nitrilases in *Ascomycota* [1] and *Basidiomycota* [2], and their use in the synthesis of analogues of the cancerostatic compound taxol [3] or (*R*)- and (*S*)-mandelic acid as synthetic building blocks [4]. A new cyanide hydratase (a nitrilase type with preference for free cyanide) was overproduced in the cells of *E. coli*, which were then used to detoxify CN^- by its conversion to formamide. The results of the nitrilase studies were reviewed [5-6]. In addition, L. Martiínková is corresponding author of solicited book chapters on this topic [7-8].
[1] *Appl. Microbiol. Biotechnol.* **100**, 2193, 2016; IF 3.376, 15 cit. [2] *Int. J. Mol. Sci.* **20**, no. 5990, 2019; IF 4.183. [3] *Org. Biomol. Chem.* **13**, 7803, 2015, IF 3.562, 8 cit. [4] *Mol. Biotechnol.* **57**, 466, 2015; IF 2.275. [5] *Appl. Microbiol. Biotechnol.* **99**, 8875, 2015; IF 3.337, 13 cit. [6] *Fung. Biol. Rev.* **33**, 149, 2019; IF 5.563, 3 cit. [7] *Green Biocatalysis*. Wiley, 2016, pp. 331-349. [8] *Science of Synthesis - Biocatalysis in Organic Synthesis 1.* Thieme, 2015, pp. 277-302.

Furthermore, we used the above strategy to search for new **aldoxime dehydratases**, which catalyze the production of nitriles from aldoximes and can be thus combined with nitrilases to form aldoxime-to-acid cascades. Aldoximes, in turn, can be readily obtained from aldehydes and hydroxylamine. We have overproduced the first aldoxime dehydratase from genus *Bradyrhizobium* and described its excellent catalytic properties such as its high activity and stability [1].
[1] (*Int. J. Biol. Macromol.* **115**, 746, 2018; IF 3.909, 2 cit.

Tyrosinases are applicable in various industrial and research areas. We have examined their applications in organic synthesis and in the biodegradation of phenolic compounds, as well as in testing melanogenesis inhibitors (tyrosinase is the key enzyme in melanogenesis). We overproduced a new fungal tyrosinase [1]. Tyrosinase was combined with cyanide hydratase for wastewater remediation [2]. The cyanide hydratase enables the action of tyrosinase by removing its inhibitor - cyanide. This process was successfully tested with real wastewaters within collaboration with iron and coking industries (Třinec Iron and Steel Works, CZ).
[1] *J. Agric. Food Chem.* **64**, 2925, 2016; IF 2.857, 9 cit. [2] *Water Research* **102**, 90, 2016; IF 5.991, 18 cit.

Metagenome-derived haloalkane dehalogenases. This is the first study describing the cloning of haloalkane dehalogenase-encoding genes from a metagenome. Total DNA was isolated from groundwater of a polluted site undergoing remediation. The full-length genes were PCR-amplified using haloalkane dehalogenase-specific primers that were previously designed in our laboratory. After cloning, the gene products were overexpressed in *Escherichia coli*. The recombinant proteins were purified and characterized in terms of their thermostability, pH and temperature optimum, quaternary structure, substrate specificity towards 30 halogenated compounds, and enantioselectivity [1]. This work was performed in collaboration with the Loschmidt Laboratories of the Masaryk University in Brno, CZ.
[1] Kotik et al., *Appl. Microbiol. Biotechnol.* **101**, 6385, 2017; IF 3.670, 2 cit.

Glycosidases active on rutin and related flavonoid glycosides. Diglycosidases are rare glycosidases, which hydrolyze the heterosidic linkage of diglycoconjugates, releasing the disaccharide and the aglycone [1]. 6-*O*-α-Rhamnosyl-β-glucosidases (rutinosidases) release the disaccharide rutinose (6-*O*-α-L-rhamnosyl-β-D-glucose) from e.g. hesperidin (hesperetin 7-*O*-rutinoside) or rutin (quercetin 3-*O*-rutinoside).

The first gene of a rutinosidase (*Aspergillus niger*) was cloned and the recombinant enzyme was used as a biocatalyst in transglycosylation reactions transferring rutinose from rutin as the glycosyl donor onto various primary (saturated and unsaturated), secondary, acyclic and phenolic alcohols. The work demonstrated the **outstanding synthetic capabilities** of this enzyme [2].

In a joint project with Prof. Breccia's laboratory (National University of La Pampa, Argentina) we identified the **first rutinosidase-encoding gene from a prokaryote** (*Actinoplanes missouriensis*). The heterologously produced protein was biochemically characterized, classified as a unique member of the glycoside hydrolase family GH55 and found to be specific towards hesperidin [3]. Unexpectedly, the chromosomal DNA of ***Acremonium*** sp. was found to encode two rutinosidases with largely different substrate specificities, one accepting only 7-*O*-β-rutinosides such as hesperidin, the other accepting also 3-*O*-β-rutinosides (*e.g.* rutin or narcissin). Both enzymes, classified as members of GH5 and GH3, were heterologously produced in *Pichia pastoris*, purified and biochemically characterized [7]. Transglycosylation reactions with hesperidin as a rutinose donor in the presence of various phenolic acceptors such as phloroglucinol, resorcinol, pyrogallol and catechol were performed using the rutinosidase from *Acremonium* sp., yielding phenolic rutinosides [6].

Several naturally occurring glucosides were enzymatically synthesized in cooperation with Sergio Riva's laboratory (CNR, Milano, IT) in one-pot reactions using the rutinosidase from *A. niger* and a rhamnosidase from *A. terreus* for the cleavage of the rhamnosyl unit from the rutinose-based transglycosylation products [4]. Transglycosylation reactions with rutin as a rutinose donor were performed in the presence of the rutinosidase from *A. niger* and various carboxylic acids (including coumaric, ferulic and caffeic acid) as acceptors. Surprisingly, rutinosylation occurred at both the phenolic hydroxyl group of the acceptor and at the carboxylic group, which is an entirely **unprecedented finding with glycosidases** [8].

A novel concept of enzymatic hydrolysis with extremely high rutin concentrations was established in our laboratory and recently published [9]. The reaction mixtures contained rutin at concentrations of up to 300 g L^{-1} (corresponding to ~0.5 M), and the reactions were performed in the absence of any organic solvents with the recombinant rutinosidase from *A. niger* as a catalyst. Full conversions were achieved although rutin and the hydrolytic product, quercetin were mostly in undissolved state. We coined these biotransformations **“solid-state” conversions**.

Finally, the **crystal structure** of the native α-L-rhamnosidase from *A. terreus* was determined in collaboration with two other Institutes of the Czech Academy of Sciences, including P. Řezáčová's laboratory (Institute of Organic Chemistry and Biochemistry). The crystallization studies were performed with the protein isolated from its natural source; thus based on the electron density map of the X-ray structure, the glycans attached to the asparagine residues could be modelled at almost all glycosylation sites [5]. We also determined the first crystal structure of the rutinosidase from *A. niger*, which served as a basis for the molecular modelling of the enzyme-rutin complex [10].

[1] Slámová et al., *Int. J. Mol. Sci.*, **19**, 2126, 2018; IF 4.183, 7 cit. [2] Šimčíková et al., *Adv. Synth. Catal.*, **357**, 107, 2015; IF 5.451, 9 cit. [3] Neher et al., *Appl. Microbiol. Biotechnol.*, **100**, 3061, 2016; IF 3.670, 8 cit. [4] Bassanini et al., *ChemSusChem*, **10**, 2040, 2017; IF 7.804, 11 cit. [5] Pachl et al., *Acta Cryst. Sect. D-Struct. Biol.*, **74**, 1078, 2018; IF 3.227, 2 cit. [6] Mazzaferro et al., *Biotechnol. Appl. Biochem.*, **66**, 53, 2019; IF 1.559, 3 cit. [7] Weiz et al., *Appl. Microbiol. Biotechnol.* 2019, **103**, 9493; IF 3.670. [8] Bassanini et al., *Adv. Synth. Catal.*, **361**, 2627, 2019; IF 5.451, VIP paper, 3 cit. [9] Kapešová et al., *Int. J. Mol. Sci.* 2019, **20**, 1112; IF 4.183, 3 cit. [10] Pachl et al., *FEBS J.* 2020, in press; IF 4.739.

New libraries of wild-type and mutant β-*N*-acetylhexosaminidases with amended catalytic properties. β-*N*-Acetylhexosaminidases from filamentous fungi are glycosidases processing *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc)-terminated glycans. They feature intrinsic transglycosylation activity, which is hampered by the concurrent hydrolysis of transglycosylation products. In order to increase yields of transglycosylation reactions, mutations in the active site of β-*N*-acetylhexosaminidase from *Talaromyces flavus* (*Tf*Hex) have been designed based on the homology model of this enzyme [1]. Three mutant variants of *Tf*Hex (Y470F, Y470H and Y470N) displayed highly increased transglycosylation activities while their hydrolytic activities were strongly suppressed [2]. These mutants are the first transglycosidases derived from a GH20 β-*N*-acetylhexosaminidase; they can be employed in an efficient biosynthesis of both natural and functionalized *N*-acetylhexosamines. Moreover, we have shown that the mutation at Y470 in *Tf*Hex changed the ratio of GalNAcase/GlcNAcase activities and that the selectivity of the mutants can be fine-tuned by reaction engineering [3]. We have also expressed and studied a new fungal β-*N*-acetylhexosaminidase from *Aspergillus versicolor* selective for GlcNAc-type substrates, applicable as an efficient biocatalyst for the synthesis of bioactive glycoconjugates [4]. Another current project involves the manipulation of the dual substrate specificity of β-*N*-acetylhexosaminidases (GlcNAc, GalNAc) through site-directed mutagenesis and preparation of selective gluco- or galactosaminidases [5]. To facilitate the evaluation of the selectivity of new inhibitors of human GH20 β-*N*-acetylhexosaminidase *vs*. GH84 *O*-GlcNAcase, a high-yielding method for the expression of the so far unavailable human HexB in the yeast expression system of *Pichia pastoris* has been elaborated [6].

In the last five years, we published two review articles on the topic of hexosaminidases, their mutagenesis and application in oligosaccharide synthesis [7-8].

[1] Kulik et al., *BMC Bioinformatics* **16**, 28, 2015, IF 2.435, 9 cit. [2] Slámová et al., *Adv. Synth. Catal.* **357**, 1941, 2015; IF 6.453, 17 cit. [3] Bojarová et al., *Molecules* **24**, 599, 2019; IF 3.060, 4 cit. [4] Bojarová et al., *Appl. Microbiol. Biotechnol.* **103**, 1737, 2019; IF 3.670, 2 cit. [5] Nekvasilová et al., *Biochim. Biophys. Acta – Proteins and Proteomics* **1868**, 140319, 2020; IF 2.540. [6] Krejzová et al., *Enzyme Microb. Technol.* **89**, 1, 2016; IF 2.502. [7] Slámová et al. *Biochim. Biophys. Acta Gen. Subj*., **1861**, 2070, 2017; IF 3.681, 12 cit. [8] Bojarová et al. *Appl. Microbiol. Biotechnol*. **103**, 7869, 2019, IF 3.670, 2 cit.

Glyco-nanomaterials and their biological activity. Our tailored synthesis of oligosaccharides and glycomimetics through methods of (chemo)enzymatic synthesis is oriented towards production of functionalized carbohydrates with desired bioactivities. Due to suitable functionalization, these compounds are then conjugated to multivalent biomaterials, which further increases the biological potential of the resulting system. We mainly address lectin targets that are either biotechnologically (wheat germ agglutinin; WGA) or biomedically (galectin-1, -3; Gal-1, -3) attractive. Thus, we have prepared the strongest glycopolymer ligands of WGA with affinities in picomolar range [1]. We furthermore prepared a range of structural types of galectin-targeting glycoconjugates that exercised their selectivity towards a particular galectin either by the choice of a suitable carbohydrate epitope [2] or by a convenient structural presentation using specific linkers [3]. Besides synthetic glycopolymers, we thoroughly studied the properties of neo-glycoproteins based on serum albumin as high-affinity galectin ligands [4-5]. We developed the concept of nature-like inert

carbohydrate linker in neo-glycoprotein conjugates, which mimics glycan presentation *in vivo*, and promotes galectin binding [6].

We developed a new method of kinetic analysis of glyco-nanomaterials by surface plasmon resonance using a specific protein construct of galectin-3 [7]. Furthermore, we published two large review articles in prestigious journals on the topic of glycol-nanomaterials and lectins [8-9].

[1] Bojarová et al. *Polym. Chem*. **8**, 2647, 2017; IF 4.930, 11 cit. [2] Bojarová et al. *J. Nanobiotechnol*. **16**, 73, 2018; IF 5.350, 6 cit. [3] Tavares et al. 2020, *Biomacromolecules* **21**, 641, 2020; IF 5,667. [4] Laaf et al. *Bioconjug. Chem*. **28**, 2832, 2017; IF 4.490, 19 cit. [5] Laaf et al. *Adv. Synth. Catal*. **359**, 4015, 2017; IF 5.646, 5 cit. [6] Laaf et al. *Adv. Synth. Catal*. **359**, 2101, 2017; IF 5.646, 14 cit. [7] Bumba et al. *Int. J. Mol. Sci.* **19**, 372, 2018; IF 4.183, 11 cit. [8] P. Bojarová and V. Křen. *Biomater. Sci*., **4**, 1142, 2016; IF 3.831, 34 cit. [9] D. Laaf et al. *Trends Biotechnol*. **37**, 402, 2019; IF 13.747, 9 cit.

Research activity and characterization of the main scientific results

During the evaluation period, the Laboratory of Cell Reproduction was reestablished. The recent team of the Lab 122 for evaluation consists of three groups of PIs J. Hasek, P. Binarova and L. Vachova. During the previous evaluation (2010 - 2014) these groups were evaluated as separate teams (Hasek - lab 122, Vachova – lab 124, Binarova – lab 144). To analyze the expression of specific proteins of interest and changes in their behavior and distribution we are using a proved approach based on a combination of useful techniques on molecular and cell biology. In biochemical, live-cell imaging and proteomics studies we use the yeast cells and plant cell lines. We construct and analyze the yeast strains and cell lines of *Arabidopsis thaliana* expressing various wild-type or mutant versions of protein fusions tagged with GFP, mRFP, mCherry, yTagRFP-T and ymTagBFP either from plasmids or from sites on the chromosomes.

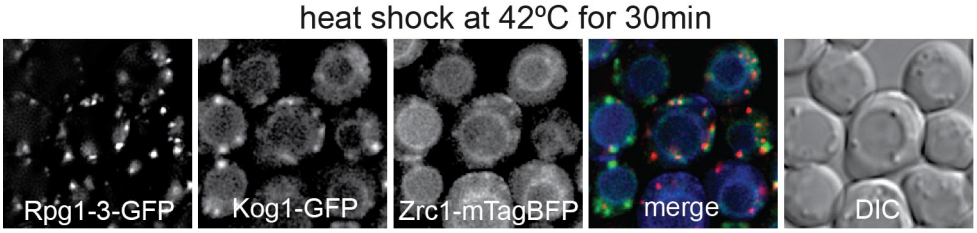
Group of J. Hasek
The main direction of research was analysis of formation and dynamics of stress granules (SGs) under various stresses in yeast *S. cerevisiae*. By testing of various mutants we searched for the answer whether formation of stress granules formed upon robust heat shock (Grousl et al. 2009) is “good or bad” for the cell survival. In this respect, our later experiments revealed that the translation initiation factor Sui2 is accumulating on the stress granules in heat stress-recovered cells (Grousl et al. 2013). This suggested that stress granules are the sites of re-initiating translation. Therefore, we suggest that ability to form stress granules is “good” for cells. We initiated a search for various mutants with affected SG dynamics. We also tested the effect of the cytoskeleton integrity. We discovered that the actin cytoskeleton in yeast *S. cerevisiae* behaves in a different way than has been published previously. This initiated a curiosity driven research on actin rearrangement in yeast cells. The aim of our work was to define changes in actin cytoskeleton during the stationary phase, the period of non-dividing state of the cell population. We found that the stationary phase cell culture contains either cells with dynamic actin cytoskeleton or cells with static actin bodies (Vasicova et al. 2015). At this point, we had to compare our data with the published knowledge, when the static actin accumulations emerge. In the literature, we found that the static accumulations of filamentous actin represent either markers of apoptosis or quiescence. Our experiments clearly revealed that actin bodies emerge in post diauxic and stationary cells with an altered mitochondrial network. The cells of this population with dynamic actin displayed active endocytosis and autophagy and well-developed mitochondrial networks. We conclude that the F-actin status reflects the extent of damage that arises from exponential growth. *Vasicova P., Lejskova R., Malcova I., Hasek J. (2015) The Stationary-Phase Cells of Saccharomyces cerevisiae Display Dynamic Actin Filaments Required for Processes Extending Chronological Life Span. Mol Cell Biol. 2015 Nov 15;35(22):3892-908. doi: 10.1128/MCB.00811-15. Epub 2015 Sep 8.*

We extended our knowledge on actin in live glucose-depleted cells by co-expressing the marker of actin patches (Abp1-RFP) with the marker of actin cables (Abp140-GFP). Glucose depletion resulted in appearance of actin patches also in mother cells. However, even after 80 min of glucose deprivation these cells showed a clear network of actin cables labeled with Abp140-GFP in contrast to previously published data. In

live cells with a mitochondrial dysfunction (rho^0 cells), glucose depletion resulted in almost immediate appearance of Abp140-GFP foci partially overlapping with Abp1-RFP patches in mother cells. Residual actin cables were clustered in patch-associated bundles. A similar overlapping "patchy" pattern of both actin markers was observed upon treatment of glucose-deprived rho$^+$ cells with FCCP (the inhibitor of oxidative phosphorylation) and upon treatment with formaldehyde. While the formaldehyde-targeted process stays unknown, our results indicate that published data on yeast actin cytoskeleton obtained from glucose-depleted cells after fixation should be considered with caution. *Vasicova P, Rinnerthaler M, Haskova D, Novakova L, Malcova I, Breitenbach M, Hasek J. (2016) Formaldehyde fixation is detrimental to actin cables in glucose-depleted* S. cerevisiae *cells. Microb Cell. 3(5):206-214. doi: 10.15698/mic2016.05.499.*

Besides a new rearrangement of actin cytoskeleton in stationary phase *S. cerevisiae* cells we discovered and described yet uknown distribution of exoribonuclease Xrn1/Kem1. Xrn1 localizes to eisosomes (MCC - Membrane Compartment of Can1) associated with the plasma membrane (in collaboration with J. Malinsky). This redistribution of Xrn1 from cytosol to MCC in early post-diauxic cells depends on eisosome structural proteins and the presence of fermentable substrate in medium. Upon heat shock, Xrn1 can still associate with heat-induced stress granules. *Grousl T., Opekarová M.., Stradalova V., Hasek J., Malinsky J. (2015) Evolutionarily Conserved 5'-3' Exoribonuclease Xrn1 Accumulates at Plasma Membrane-Associated Eisosomes in Post-Diauxic Yeast PLoS One. Mar 26;10(3):e0122770. doi: 10.1371/journal.pone.0122770. eCollection 2015*

We also continued in elucidation of stress-induced rearrangement of translation machinery in various yeast mutants. To obtain more data we had to establish a new system of microscopic investigation of three fluorescently tagged fusion protein in live yeast cells. An important point of this task was to use additional and new markers to save the old markers for other genetic manipulations the specific strains. Using this approach, we could perform complicated experiments with microscopic investigation of various fusion proteins in various specific mutants. The creation of these suitable tools we published much later in 2016 (Malcova et al. 2016). Created collection of nine DNA integrative modules for the construction of new strains of the yeast *Saccharomyces cerevisiae* is suitable for C - terminal labeling with fluorescent proteins yTagBFP, mCherry and yTagRFP-T in connection with three different selection antibiotics: geneticin, nourseothricin and hygromycin. In combination with the collection of strains producing GFP-fusion proteins from sites on the chromosome, our technique allows us to observe the distribution of up to three proteins simultaneously in living cells. Free genetic markers serve to manipulate other genes in the labeled strains. All the constructs were made available to the community through Addgene, a nonprofit plasmid repository or on personal requests. Since 2016 there was 47 requests for requests for various cassettes and/or plasmids have already been served. The paper was selected as Editor´s choice for the issue of FEMSYR. *Malcova I., Farkasovsky M., Senohrabkova L., Vasicova P., Hasek J. (2016) New integrative modules for multicolor-protein labeling and live-cell imaging in Saccharomyces cerevisiae. FEMS Yeast Research, 2016 May;16(3). pii: fow027. doi: 10.1093/femsyr/fow027. Epub 2016 Mar 17.*



Cells have elaborated a complex strategy to maintain protein homeostasis under physiological as well as stress conditions with the aim to ensure the smooth functioning of vital processes and producing healthy offspring. Impairment of one of the most important processes in living cells, translation, might have serious consequences including various brain disorders in humans. Here, we describe a variant of the translation initiation factor eIF3a, Rpg1-3, mutated in its PCI domain that displays an attenuated translation efficiency and formation of reversible assemblies at physiological growth conditions. Rpg1-3-GFP assemblies are not sequestered within mother cells only as usual for misfolded-protein aggregates and are freely transmitted from the mother cell into the bud although they are of non-amyloid nature. Their bud-directed transmission and the active movement within the cell area depend on the intact actin cytoskeleton and the related molecular motor Myo2. Mutations in the Rpg1-3 protein render not only eIF3a but, more importantly, also the eIF3 core complex prone to aggregation that is potentiated by the limited availability of Hsp70 and Hsp40 chaperones. Our results open the way to understand mechanisms yeast cells employ to cope with malfunction and aggregation of essential proteins and their complexes. *Senohrabkova L, Malcova I, Hasek J. (2018) An aggregation-prone mutant of eIF3a forms reversible assemblies escaping spatial control in exponentially growing yeast cells. Curr Genet. 65(4):919-940. doi: 10.1007/s00294-019-00940-8. Epub 2019 Feb 4.*

We described previously that translationally controlled tumor protein (TCTP), that is a multifunctional and highly conserved protein from yeast to humans, is also localized to stress granules in heat stressed cells (Rinnerthaler et al. 2013). Recently, its role in non-selective autophagy has been reported with controversial results in mammalian and human cells. Herein we examine the effect of Mmi1, the yeast ortholog of TCTP, on non-selective autophagy in budding yeast *Saccharomyces cerevisiae*, a well-established model system to monitor autophagy. We induced autophagy by nitrogen starvation or rapamycin addition and measured autophagy by using the Pho8Δ60 and GFP-Atg8 processing assays in WT, *mmi1Δ*, and in autophagy-deficient strains *atg8Δ* or *atg1Δ*. Our results demonstrate that Mmi1 does not affect basal or nitrogen starvation-induced autophagy. However, an increased rapamycin-induced autophagy is detected in *mmi1Δ* strain when the cells enter the post-diauxic growth phase, and this phenotype can be rescued by inserted wild-type *MMI1* gene. Further, the *mmi1Δ* cells exhibit significantly lower amounts of reactive oxygen species (ROS) in the post-diauxic growth phase compared to WT cells. In summary, our study suggests that Mmi1 negatively affects rapamycin-induced autophagy in the post-diauxic growth phase and supports the role of Mmi1/TCTP as a negative autophagy regulator in eukaryotic cells. *Vojtova J, Hasek J.Mmi1, the Yeast Ortholog of Mammalian Translationally Controlled Tumor Protein (TCTP), Negatively Affects Rapamycin-Induced Autophagy in Post-Diauxic Growth Phase. Cells 9(1):138. doi: 10.3390/cells9010138.*

We were also able to demonstrate that Mmi1, similarly to other specific proteins, shuttle between LDs and mitochondria. It depends on the metabolic state of the cell on which organelle these proteins are predominantly localized. Responsible for the localization of the particular proteins is a protein domain consisting of two *α*-helices, which we termed V-domain according to the predicted structure. So far we have detected this domain in the following proteins: mammalian BAX, BCL-XL, TCTP and yeast Mmi1p and Erg6p. According to our experiments, there are two functions of this domain: (1) shuttling of proteins to mitochondria in times of stress and apoptosis; (2) clearing the outer mitochondrial membrane from pro- as well as anti-apoptotic proteins by moving them to LDs after the stress ceases. In this way the LDs are used by the cell to modulate stress response. *Bischof J, Salzmann M, Streubel MK, Hasek J, Geltinger F, Duschl J, Bresgen N, Briza P, Haskova D, Lejskova R, Sopjani M, Richter K, Rinnerthaler M. (2017) Clearing the outer mitochondrial membrane from harmful proteins via lipid droplets. Cell Death Discov. 3:17016. doi: 10.1038/cddiscovery.2017.16. eCollection 2017.*

To analyze the effect of stress and stress granule formation on the cell polarity and the apical growth we underwent into study of yeast casein kinases using the specific *yck2* ts mutant. We found that the absence of Phospholipase C (Plc1p) blocks the apical growth of *yck2*Δ *yck1 ts* mutant. Phospholipase C (Plc1p) is also required for normal localization of glucose transporters to the cell membrane. Consequently, *plc1*Δ cells display histone hypoacetylation and transcriptional defects due to reduced uptake and metabolism of glucose to acetyl-CoA, a substrate for histone acetyltransferases. In the presence of glucose, Mth1p is phosphorylated by casein kinase I Yck1/2p, ubiquitinated by the SCFGrr1 complex and degraded by the proteasome. Here, we show that while Plc1p does not affect the function of the SCFGrr1 complex or the proteasome, it is required for normal protein level of Yck2p. Since stability of Yck1/2p is regulated by a glucose-dependent mechanism, *PLC1* inactivation results in destabilization of Yck1/2p and defect in Mth1p degradation. *Zhang T, Galdieri L, Hasek J, Vancura A. (2017) Yeast phospholipase C is required for stability of casein kinase I Yck2p and expression of hexose transporters. FEMS Microbiol Lett. 364(22):fnx227. doi: 10.1093/femsle/fnx227.* We used this model system and we found that Plc1 is also required for localization of Hrr25 to the plasma membrane to induce the apical growth (Dohnalek, Diploma thesis).

Group of L. Vachova

In early 2016, the research group of L. Vachova relocated to the newly constructed, well equipped Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University in Vestec (BIOCEV). Within BIOCEV, L. Vachova was responsible for collaboration on the project 2.1.6: “Role of Metabolism, Signaling Molecules and Cellular Structures in the Process of Aging, Stress and Adaptation” with the “Yeast colony group” (YCG) of Z. Palková from the Faculty of Science, Charles University in Prague (FoS CUNI). Both groups are now internationally recognized in the field of yeast multicellularity. This is a highly complex research area, covering a wide spectrum of different cellular processes and requiring a wide spectrum of techniques (including OMICS methods and state-of-the-art microscopy) that are well established and distributed between the groups. The two groups have jointly developed unique techniques of colony sample preparation, enabling in situ study of cellular processes

within the colony structure, using state-of-the-art two-photon confocal microscopy (2PE-CM) or using separated cell subpopulations.

One of the most important discoveries of the evaluated period was the identification of mitochondria and mitochondral retrograde (RTG) signaling as key players in the regulation of U/L cell differentiation and metabolic reprogramming during colony development (Podholova et al., *Oncotarget* 7:15299, 2016; Palkova and Vachova, *Aging* 8: 1287, 2016; Wilkinson et al., *Oxid Cell Med Longev* 2018: ID 4932905. 2018). The previously identified RTG pathway includes three activators, Rtg1p, Rtg2p and Rtg3p, and several repressors including Mks1p and TORC1 (Liu and Butow, *Annu Rev Genet* 40:159, 2006); studies in liquid cultures showed anaplerotic pathway to be regulated by RTG signaling with *CIT2* as one of the major targets. In colonies we identified three specific RTG branches that divergently regulate the properties and fate of three specifically localized cell subpopulations through signals from differently altered mitochondria: i) ROS-free mitochondria activate the "Ato-branch" of RTG signaling in low respiring U cells, leading to activated expression of *ATO1* and *ATO2* genes, involved in ammonia export and metabolic reprogramming of these cells; this branch is active despite TORC1 (described as a repressor of RTG signaling in yeast liquid cultures) being active, ii) the "Cit2p-branch" which is active in upper layers of L cells and activates expression of the *CIT2* gene and related metabolic reprogramming and iii) the “cell viability branch” which is active in lower L cells where functional RTG signaling is essential for cell viability. All three branches require all activators, Rtg1p, Rtg2p and Rtg3p, as well as repressor Mks1p. The important role of mitochondria in colony differentiation was further supported by analysis of transcriptome remodeling in small subpopulations of differentiated cells. Altogether our novel findings showed that the roles of RTG signaling in yeast are far more complex, and affect more cellular processes, than hitherto anticipated and are involved in a number of, as yet unknown, regulatory elements - thus resembling pleiotropic mitochondrial retrograde signaling in mammals. Identification of a new RTG signaling branch in U cells could be particularly important, because these cells reprogram and naturally downregulate mitochondrial respiration. Based on this finding we are currently trying to identify regulators upstream of Rtg factors involved in activation of this branch (i.e., potential signaling between mitochondria and Rtg2p) as well as those that target Rtg1p/Rtg3p to specific genes in U cells. Experimental work was split between the collaborating groups (LV and YCG) and model schemes were developed together using complementary know how. For example, LV group performed analyses of protein abundances and levels of mRNAs of RTG targets and contributed to analyses of cellular localization of RTG targets in colony cell subpopulations. YCG, for example, prepared all strains and performed experiments on giant colonies and OMICS analyses.

Other important findings on differentiated colonies reached during the evaluated period include:
i) Identification of the important role of glycolysis in U cell longevity in differentiated colonies (Cap et al., *Cell Cycle*, 14: 3488, 2015). In this paper, we also showed that the glyoxylate cycle and gluconeogenesis are active in L cells and identified degradation of L cell wall material as a possible source of sugars for U cell-specific glycolysis. These findings further supported a model of nutrient flow between U and L cells. Members of LV group participated in analyzing the effect of glycolysis inhibitors on U cells by 2PE-CM, in experimental design and in writing of the paper.

ii) Participation in uncovering the regulation of hexose transporters and their degradation by endocytosis (Hovsepian et al. *J Cell Biol* 216:1811, 2017). Together with YCG, we preformed all experiments related to the regulation of hexose transporter Hxt6p homeostasis by glucose availability in the context of yeast colonies. This finding together with other, as yet unpublished data on other hexose transporters, revealed which transporters are responsible for glucose uptake by U cells. The French partner uncovered principles of endocytosis control and performed all experiments regarding this regulation in liquid cultures.

An important discovery was also the identification of a new regulatory mechanism of biofilm-specific processes and formation of complex colony biofilm structure (Nguyen Van et al., *PLOS Genetics*: 14:e1007495, 2018; Vachova and Palkova, *Curr Genet*. 65:147, 2019). Two transcription regulators Cyc8p and Tup1p were shown to antagonistically regulate colony biofilms. Cyc8p functions as a repressor of *FLO11* expression (Flo11p adhesin is indispensable for biofilm formation) and of biofilm formation, including cell invasiveness into the agar, whereas Tup1p counteracts the Cyc8p repressor function and positively regulates biofilm formation and Flo11p expression

Discovery of Cyc8p/Tup1p regulation of colony biofilms formed a basis for identifying the important role of these regulators in the developmental cycle of true biofilms, formed at solid-liquid interface (Nguyen Van et al., *npj Biofilms and Microbiomes* 6:7, 2020). Cyc8p and Tup1p antagonistically regulate two phases of formation of solid liquid interface biofilms - cell adhesion (and formation of biofilms structure) and release of planktonic cells. Furthermore, we discovered that glucose regulates Cyc8p level and thus the switch between cell adhesiveness and release of planktonic cells from the biofilm.

Our knowledge of cell death in yeast populations led to participation, together with world-leading experts, in a critical evaluation of fungal ability to undergo caspase-dependent apoptosis-like cell death (Aouacheria et al., *Science* 360:eaar6910, 2018). This critical evaluation is based on the fact that many features of yeast regulated cell death are not similar to features of mammalian apoptosis. Furthermore, various methods used to identify apoptosis in mammals, are producing artifacts when used in fungi (for example, detection of caspases by inhibitor FITC-VAD-FMK as we showed in the past).

The importance of our research into yeast multicellularity to other groups worldwide can be demonstrated by numerous invitations to write review papers discussing different aspects of yeast multicellularity (Palkova and Vachova, *Semin Cell Dev Biol*. 57: 110, 2016; Vachova and Palkova, *FEMS Yeast Res*. 18:foy033, 2018; Palkova and Vachova, *Aging* 8: 1287, 2016; Vachova and Palkova, *Curr Genet*. 65:147, 2019).

Applied research
We designed a new copper biosensor based on an immobilized, modified yeast strain (Vopalenska et al., *Biosensors and Bioelectronics* 72:160, 2015). The biosensor uses a *S. cerevisiae* strain with the *ADE2* gene deleted from the genome and the natural promoter regulating the expression of the *ADE5,7* gene replaced with the *CUP1* promoter. The resulting strain produces red pigment only in the presence of Cu^{2+} and in a quantity proportional to copper concentration in the range 1–100 μM. The intensity

of the red color, and therefore of Cu^{2+} detection, are optimal when the strain is immobilized. The strain modification process has been patented (Patent document 305223, 2015).

Group of P. Binarova

is focused to molecular biology of cytoskeleton specifically to gamma-tubulin. Gamma-tubulin, in addition to well-established role in microtubule nucleation, shows a number of important but less understood cellular functions. The team occupies a rather noticeable position in the field based on continuity and consistency of the research. Recently we characterized large molecular forms of gamma-tubulin in plant and animal cells and the ability of the protein to assemble fibrilar oligo/polymers. We aimed to understand functions of gamma-tubulin in nuclei, specifically in processes of transcription and repair:

We found that mitogen-activated protein kinase MPK6 interacts with gamma-tubulin and mitotic defects were observed under reduction of the kinase activity. While either gamma-tubulin or gamma-tubulin complex protein GCP4 were not phosphorylated by MPK6, a microtubule plus-end protein EB1c was. Our data suggest a scaffolding role of gamma-tubulin in MAP kinase signaling and help to understand role of MPK6 in plant cells. Experiments, manuscript writing, the first and corresponding author our team (80 %). P.Halada MS data, S Nagy, T. Mészáros in vitro translations, 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. (2015). The Arabidopsis mitogen-activated protein kinase 6 is associated with γ-tubulin on microtubules, phosphorylates EB1c and maintains spindle orientation under nitric oxide stress. New Phytol., Sep; 207(4):1061-74.*

We found that RBR and E2FA formed foci at sites of DNA damage predominantly in heterochromatin rich pericentromeric region. Process was ATM and ATR dependent and BRCA1 potein colocalized partially with RBR/E2F foci. RBR homologue of pRb in *Arabidopsis* was shown to function in DNA damage response and genome maintenance. H. Kourova the first author performed experiments in our laboratory, P. Binarova contributed to design of experiments, data evaluation, and manuscript writing. *Horvath, B#., Kourova, H.,# Nagy, S., Nemeth, S., Magyar, Z., Papdi, C., Ahmad, Z., Sanchez-Perez G., Perilli S., Blilou, I., Meszaros, T., Binarova, P., Bogre, L., B. Scheres. (2017). Arabidopsis RETINOBLASTOMA RELATED regulates DNA damage response independently of the cell-cycle. EMBO J., May 2; 36(9): 1261–1278. Mar 20. doi: 10.15252/embj.201694561, # H.* Kourova shared first authorship

We demonstrated ability of plant and animal gamma-tubulin to form oligo/polymers and we characterized transition between fibrilar and aggregated forms of gamma-tubulin. Superresolution microscopy showed gamma-tubulin fibrillar structures in plant cells localized with microtubules, at perinuclear area and in nuclei. Our finding that gamma-tubulin belongs to filament forming tubulins help understanding multiple cellular functions of the protein besides its well established role in microtubule nucleation. Experiments, manuscript writing our team with the first and corresponding author (75%). P.Halada MS data, G. Daniel TEM analyses, V. S., A. K. and P. D. purified mammalian g-tubulin. *Chumová, J., Trögelová, L., Kourová, H., Volc, J., Sulimenko, V., Halada, P., Kučera, O., Benada, O., Kuchařová, A., Klebanovych, A., Dráber, P.,*

Daniel, G., Binarová P. # (2018). gamma-Tubulin has a conserved intrinsic property of self-polymerization into double stranded filaments and fibrillar networks. Biochim Biophys Acta. Molecular Cell Research 2018 Feb 27;1865(5):734-748. doi: 10.1016/j.bbamcr.2018.02.009

We found that gamma-tubulin linear oligomers/polymers are present also with the outer and the inner nuclear envelope in Arabidopsis cells. We showed that plant gamma-tubulin interacts with LINC complex protein SUN. Data on nuclear functions of gamma-tubulin are discussed in the light of our finding of the protein polymerization and its interactions on inner nulear periphery and with SUN protein. Experiments, manuscript writing were performed by our team with the first and corresponding author (85%). P. Halada provided MS data. *Chumová, J., Kourová, H., Trögelová, L., Halada, P., Binarová, P. (2019). Microtubular and Nuclear Functions of -Tubulin: Are They LINCed? Cells. 2019 Mar 19;8(3). 1-17. pii: E259. doi: 10.3390/cells8030259.*

Altogether our data demonstrated ability of gamma-tubulin to assemble filaments present also with microtubules and in nuclei. We found that gamma-tubulin provides a platform for interaction with MAP kinases and interacts with LINC complex protein SUN.. Gamma-tubulin also partially colocalized with RBR/E2F foci formed at sites of DNA damage response in centromeric region at nuclear periphery. Consistently, our the most recent data showed that plant gamma-tubulin with E2F transcription factors associates with the promoters of E2F-regulated genes. *Kállai B.M., Kourová H., Chumová J., Papdi C., Trögelová L., Kofroňová O., Hozák P., Filimonenko V., Mészáros T., Magyar Z., Bögre L., Binarová P.[1]#. γ-Tubulin interacts with E2FA, E2FB and E2FC transcription factors, regulates proliferation and endocycle in Arabidopsis. Journal of Experimental Botany, Vol. 71, No. 4 pp. 1265–1277, 2020 doi:10.1093/jxb/erz498. Advance Access Publication November 6, 2019. IF 2019 = 5.908).*

Research activity and characterisation of the main scientific results

Topic 1 - APD compounds

Most natural products synthesized in microbial secondary metabolism pathways are complex compounds consisting of several building blocks. These blocks can be regular intermediates supplied by primary metabolism, however, more often, several unusual precursors synthesized in specialized biosynthetic pathways are assembled into the final natural product. Additionally, each individual unusual precursor can be incorporated into several different structural contexts, i.e., it can be combined with distinct types of building blocks, resulting in complex natural products from structurally diversified families, with different targeting biological structures, and thus with different biological function.

4-Alkyl-L-proline derivatives (APDs) represent an example of a rarely occurring specialized building block exclusive for some actinomycete complex natural products only, so called **APD compounds** (Janata et al., 2018). Until our invited review, only three structurally and functionally diverse groups of natural products were known to incorporate APD precursors: antitumour agents pyrrolobenzodiazepines, signalling molecule hormaomycin and antibiotic lincomycin. **We postulated general principles of biosynthesis and incorporation of APD precursors**, including marker genes and common features of their **biosynthetic gene clusters (BGC)**. Using these rules we **found more than 40 putative BGC encoding new APD compounds**, including yet uknown groups. Up to now, we expanded the number of putative novel BGCs encoding APD compounds to more than 100, including brand new classes of with yet uncovered types of biological activities. We hypothesize (based on known examples), the APD compounds generally represent evolutionary more advantageous variants of their respective L-proline incorporating ancestors. Moreover, we **suggested the unification of nomenclature of six APD biosynthetic proteins** (Apd1 to Apd6), reflecting their sequence in APD biosynthesis, which has been accepted in overview on biosynthesis of non-proteinogenic α-amino acids (Hedges an Ryan, *Chemical Reviews*, 120, 3161−3209, 2020)

The APD story started already in 2012, when we revealed that the scheme of the APD precursor biosynthesis, previously proposed in the literature, was incorrect and we postulated a new scheme based on indirect evidence (Jiraskova et al., 2016).

Subsequently, we have supported or confirmed several of the postulated hypotheses for the biosynthetic steps. First, we have shown evidence based on experimental data and homology models that the C-C bond cleavage in APD biosynthesis cannot precede the C-methylation step (Kamenik et al., 2018), bringing an important point to the debate on the remarkable discovery of the N-terminal nucleophile-hydrolases. Second, we conducted experiments with recombinant proteins and elucidated the final step of APD biosynthesis, catalyzed by F420-dependent reductases (Steiningerova et al., 2020). This reaction is particularly intriguing because homologous reductases from related pathways showed different reaction specificities (reduction of one vs. two double bonds of the same substrate), providing APD precursors and thus final molecules with optimal shapes to bind their distinct biological targets (50S bacterial ribosome for lincosamides and minor groove of DNA for pyrrolobenzodiazepines). We have also investigated

incorporation of the APD units into the natural compounds. Specifically, we documented the evolutionary adaptation of a lincosamide adenylation domain with strict substrate specificity for L-proline towards the acceptance of APD, a sterically different substrate (Vobruba et al., 2017). We also elucidated how pyrrolobenzodiazepines, another group of APD compounds, are assembled, revealing that the diversity of their APD moieties are a result of post-condensation modifications (Kamenik et al., 2017). Another contribution to the biosynthesis of pyrrolobenzodiazepines is the discovery of a novel pathway for the formation of 3-hydroxyanthranilic acid in the biosynthesis of limazepines (Pavlikova et al., 2018).

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Pavlikova, M., Kamenik, Z., Janata, J., Kadlcik, S., Kuzma, M., and Najmanova, L.* (2018). Novel pathway of 3-hydroxyanthranilic acid formation in limazepine biosynthesis reveals evolutionary relation between phenazines and pyrrolobenzodiazepines. *Sci. Rep.* 8, 1–10.

** All publications were corresponded by members of our group.*

Topic 2- Natural and hybrid lincosamides

The biosynthesis of structurally **the most exciting APD compound, antibiotic lincomycin**, where the APD is attached to another specialized precursor, an amino thio-octose, was clarified almost twenty years after the publication of its BGC and also with our participation. The **unique lincomycin condensation system** combines nonribosomal peptide synthetase (NRPS) components that are responsible for APD activation (Janata et al., 2015) with unique NRPS-dissimilar ergothioneine-mycothiol dependent activities, responsible for amino sugar conjugation/activation and subsequent amide bond formation which together result in sulphur-atom incorporation into the lincosamide structure (Zhao et al., *Nature*, *518*(7537), 115-119, 2015).

The discovery of an amazing condensation system coupled with metabolism of two small-molecule thiols allowed us **to design and demonstrate remaining post-condensation steps in lincomycin biosynthesis** (Kamenik et al., 2016), including postulation of the biosynthetic branch-point that directs whether the attachment of another building block, salicylic acid in celesticetin biosynthesis, will occur. Celesticetin is another natural lincosamide antibiotic, less efficient than lincomycin, where proteinogenic L-proline (instead specialized APD) is attached to the thio-octose

lincosamide core unit but also with the additional salicylate unit attached to its sulphur-atom by two-carbon linker. This elucidation of biosynthetic principles **opened the door to the biological preparation of hybrid lincosamide compounds combining features of lincomycin** (APD unit) **and celesticetin** (additional salicylate unit). More than 100 hybrid lincosamide compounds has been prepared. Two of them, **CELIN** (CElesticetin-LINcomycin) and its O-demethyl derivative **ODCELIN were more efficient if compared to industrially produced lincomycin** (Kadlcik et al., 2017)**,** probably due to extended surface available for interaction between the antibiotic and its targeting structure, the peptidyl transferase centre (PTC) of the 50S ribosomal subunit (**i.e. by improved molecular fitting**). As expected, the chlorinated derivative (Cl-ODCELIN) was even more efficient if compared to CELIN and OD-CELIN. **Both CELIN derivatives as well as their chlorinated derivatives are now subject of international patent application** (Janata et al., 2017).

Research for practise

Lincomycin and its chlorinated semisynthetic derivative clindamycin are clinically important antibiotics; in the USA, clindamycin belongs among TOP 10 most prescribed antibiotics, also due to its excellent penetration deeply into tissues, including bone (often prescribed against anaerobic infections). However, there are two limits threatening the position of clindamycin in the TOP10: First, *Clostridioides difficile* is the causative agent of **pseudomembranous colitis**. The colitis is a condition resulting from previous antibiotic treatment (**antibiotic-associated colitis), including clindamycin**. In the USA alone, colitis kills about 30 000 patients every year, it is the most common fatal infection complication not only in hospitals but also in nursing homes and similar facilities. Second, for a long time, clindamycin represented an effective alternative to penicillins against staphylococcal and streptococcal infections including those caused by **methicillin-resistant *S. aureus* (MR; MRSA**), one of the most severe pathogens from the WHO list of the 12 most dangerous pathogens. However due to increasing co-occurrence of MR and methylation of adenine nucleotide in PTC, clindamycin more and more often fails against MRSA.

When compared to currently marketed lincosamide drugs, ODCELIN and Cl-ODCELIN are more efficient against staphylococci, streptococci and other relevant bacteria, including anaerobes and exhibit a broader spectrum of antimicrobial activity (Table). The extremely low MIC (0.03 µg/ml) is promising even for the treatment of chronic staphylococcal infections. Both tested CELIN derivatives show promising activity against some MRSA and other MR pathogens and even against *Enterococcus faecium* (from the WHO list), which is intrinsically resistant to commercial lincosamides. **Most importantly, Cl-ODCELIN is highly efficient against *Clostridioides difficile,* including the highly-resistant strains.**

Last year we have developed in **cooperation with the small Czech company Santiago** chemikálie s.r.o. (specialized on chemical synthesis) a method for the chemical synthesis of ODCELIN and its chlorinated derivative Cl-ODCELIN in multi-gram scale. In 2020, we submitted a joint grant application to Technology Agency of the Czech Republic and started a preclinical testing of Cl-ODCELIN.

		Minimal inhibitory concentrations (mg/l)			
		„Natural“		Chlorinated	
Strain	RESISTANCE	LINCOMYCIN	ODCELIN	CLINDAMYCIN	CL-ODCELIN
Staphylococcus aureus A6989	MRSA, inducible MLS_B	2	0.25	0.5	0.031
Staphylococcus aureus 73OL	MRSA, constitutive MLS_B	≥2048	2	256	0.125
Staphylococcus aureus MU50	MRSA, constitutive MLS_B	≥2048	256	512	32
Staphylococcus haemolyticus 53-67	-	0.25	0.031	0.25	0.031
Staphylococcus epidermidis 46	-	2	0.125	0.25	0.031
Staphylococcus epidermidis 19	Constitutive MLS_B	≥2048	256	>2048	128
Clostridium difficile 4966	-	32	2	8	0.063
Clostridium difficile 4968	Constitutive MLS_B	≥2048	256	512	1
Streptococcus pneumoniae 27775	Constitutive MLS_B	128	32	128	0.125
Enterococcus faecium E1162	no data	512	0.125	32	0.063
Enterococcus faecium E1105	no data	≥2048	256	>2048	32

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** All publications were corresponded by members of our group.*

Topic 3 - Antibiotic resistance and ABCF-mediated antibiotic signaling cascade

During the 2015-2019 evaluation period, the Gabriela Balikova Novotna group studied the clinical aspects and mechanisms of resistance to 50S ribosomal subunit-targeting antibiotics including lincosamides and cell wall-targeting glycopeptides. We employed molecular microbiology and omics approaches to understand the function of the resistance proteins, the consequences of their activity in the cell, and genetic pathways leading to the development of resistance by mutations. The work was supported by two grants (AZV 15-28807A and GACR 15-16225Y). In the first project, we studied antibiotic resistance proteins (ARE) of the ABC family F (ABCF). The hypothesis of the ribosomal resistance mechanism postulated by us at the end of the last evaluation period (Lenart et al., 2015) was shortly after publication proved by (Sharkey et al., (2016) ABC-F proteins mediate antibiotic resistance through ribosomal protection. mBio 7, e01975-15) and was followed by several structural studies, confirming the common ribosomal mode of functioning of a whole ABCF family as modulators of ribosomal PTC. Our work on the biological function of ARE ABCF from the lincomycin

biosynthetic gene cluster came with the **groundbreaking finding that** a substantial part of **bacterial ABCF proteins**, which were previously classified as purely antibiotic resistance proteins has **in addition to resistance the antibiotic signal-transducing function regulating gene expression in response to** 50S ribosomal-targeting **antibiotics**. Particularly, we demonstrated that the dual character of ABCF ATPase LmrC confers antibiotic resistance, and simultaneously transduces a signal from ribosome-bound antibiotic to transcription of lincomycin biosynthetic gene cluster-specific regulator (Koberska et al., 2020, revising for mBio). Our work suggests that these proteins might form analogous signal transduction systems participating not only in the autoregulation of biosynthesis but probably also in the inter-cellular antibiotic signaling and stress response induction. Given the number and diversity of small molecules targeting the 50S ribosomal subunit and the number of bacterial ABCF proteins encoded by soil bacteria from the Terrabacteria group, which includes Firmicutes and Actinobacteria with the highest number of ABCF per genome, **ABCF-mediated signaling could be one of the most important tools of chemical communication in general**.

In the second project, using comparative whole-genome sequencing of the wild-type and mutant *S. epidermidis* and *S. haemolyticus* strains of different clades we demonstrated that the genetic basis of glycopeptide resistance development differs between these two species and that *S. haemolyticus* is more prone to develop the resistance. In addition, we have found that membrane proteins from the VanZ family that are encoded in genomes of gram-positive pathogens outside the glycopeptide resistance gene clusters represent a thread to the new generation of lipoglycopeptides (Vimberg et al., 2020). GBN group also collaborated on the development of new antimicrobials overcoming the resistance. We developed a fluorescent assay that predicts in vitro antibacterial activity of the novel glycopeptide antibiotics designed by Prof. Pal Herzegh (Vimberg et al., 2019) and we evaluated the hydnocarpin derivatives developed by Radek Gažák as potent inhibitors of staphylococcal biofilm formation (Vimberg et al., 2015).

References:

Lenart, J., Vimberg, V., Vesela, L., Janata, J., and Novotna, G. B.* (2015). Detailed mutational analysis of Vga (A) interdomain linker: Implication for antibiotic resistance specificity and mechanism. *Antimicrob. Agents Chemother.* 59, 1360–1364.

Koberska, M., Vesela, L., Vimberg, V., Lenart, J., Vesela, J., Kamenik, Z.,…Janata J., and Novotna, G. B.* (2020). Beyond self-resistance: ABCF ATPase LmrC is a signal-transducing component of an antibiotic-1 driven signaling cascade hastening the onset of lincomycin biosynthesis, bioRxiv, 2020.10.16.343517. doi:10.1101/2020.10.16.343517, under revisions in *mBio*.

Vimberg, V., Kuzma, M., Stodůlková, E., Novák, P., Bednárová, L., Šulc, M., & Gažák, R.* (2015). Hydnocarpin-type Flavonolignans: semisynthesis and inhibitory effects on Staphylococcus aureus biofilm formation. Journal of natural products, 78(8), 2095-2103.

Vimberg, V., Gazak, R., Szűcs, Z., Borbás, A., Herczegh, P., Cavanagh, J. P., ... & Novotna, G. B.* (2019). Fluorescence assay to predict activity of the glycopeptide antibiotics. *The Journal of antibiotics*, 72(2), 114-117.

Vimberg, V., Zieglerová, L., Buriánková, K., Branny, P., & Balíková Novotná, G.* (2020). VanZ Reduces the Binding of Lipoglycopeptide Antibiotics to Staphylococcus aureus and Streptococcus pneumoniae Cells. *Frontiers in Microbiology*, 11, 566.

** All publications were corresponded by members of our group*

Topic 4 - Metabolomics

For the previous topics 1-3 (biosynthesis of specialized metabolite and antibiotic resistance), we used chemical analysis as one of the key methodological approaches. In addition, we exploited our expertise in this field for extensive collaborations. Specifically, we were involved in the studies on the plant phytotoxic metabolites (Jandová et al., 2015), metabolism of bioactive chemicals in the brain (Valny et al., 2016), specialized metabolites of *Streptomyces coelicolor* (Čihák et al., 2017), and low-molecular-weight compounds in soil (Buresova et al., 2019). Given the relatedness of these collaborative topics to the research interest of our group, we not only performed the chemical analysis, but we also participated in the biological interpretation and significance of the data. Our expertise in analytical chemistry was predominantly focused on targeted analysis (**targeted metabolomics**). However, more complex research questions require **untargeted metabolomics**, which is one of the approaches of the OMICS ´pipeline´used nowadays to describe complex living systems. The expertise in non-targeted mass spectrometry was obtained during a **six-month-visit of our member, Zdenek Kamenik, in the research group of Pieter Dorrestein, University of California at San Diego, USA (autumn-winter 2018).** During this stay, he participated in the development of several new tools for mass spectrometry-based metabolomics, which have so far resulted in two publications (published in 2020 in Nature Methods), and another manuscript submitted in Nature Communications. This is the **first platform that relies on an innovative algorithm that compares MS/MS spectra within a dataset and results in molecular networks, in which related compounds are clustered together and can be visualized with metadata** (e.g. different species, wild type vs. mutant strain, healthy person vs. patient, different treatments, nutrition, diets, culture media composition, time-course experiments, etc.). Metadata give the important context to the LC-MS&MS/MS data, facilitating data mining to confirm a hypothesis, draw a new one, or simply get a scientifically relevant information. Importantly, the workflow also includes in silico and statistical tools, and it enables compound annotation using community-based and third party-derived GNPS libraries with more than 200 thousands MS/MS spectra.

Subsequently, Zdenek Kamenik combined the expertise in non-target metabolomics and a new liquid chromatography-mass spectrometry instrument acquired at IMIC, and elaborated a research program in mass spectrometry-based microbial metabolomics, which **received a financial support of the Czech Academy of Sciences within the five-year-long prestigious grant Lumina Quaeruntur, starting in 2020.**

References:

Buresova, A., Kopecky, J., Hrdinkova, V**., Kamenik, Z**., Omelka, M., and Sagova-Mareckova, M. (2019). Succession of microbial decomposers is determined by litter type, but site conditions drive decomposition rates. *Appl. Environ. Microbiol.* 85.

Čihák, M., **Kameník, Z**., Šmídová, K., Bergman, N., Benada, O., Kofronová, O., et al. (2017). Secondary metabolites produced during the germination of Streptomyces coelicolor. *Front. Microbiol.* 8, 2495.

Jandová, K., Dostál, P., Cajthaml, T., and **Kameník, Z.** (2015). Intraspecific variability in allelopathy of Heracleum mantegazzianum is linked to the metabolic profile of root exudates. *Ann. Bot.* 115, 821–831.

Valny, M., Honsa, P., Kirdajova, D., **Kamenik, Z.**, and Anderova, M. (2016). Tamoxifen in the mouse brain: Implications for fate-mapping studies using the tamoxifen-inducible cre-loxP system. *Front. Cell. Neurosci.* 10, 243.

Jarmusch, A. K., Wang, M., Aceves, C. M., Advani, R. S., Aguirre, S., Aksenov, A. A., ...**Kamenik, Z**.,…. & Dorrestein, P. (2020). ReDU: a framework to find and reanalyze public mass spectrometry data. *Nature Methods*, 17(9), 901-904.

Nothias, L. F., Petras, D., Schmid, R., Dührkop, K., Rainer, J., Sarvepalli, A., ... **Kamenik, Z.**,…. & Dorrestein, P. (2020). Feature-based molecular networking in the GNPS analysis environment. *Nature Methods*, 17(9), 905-908.

Research activity and characterization of the main scientific results

1. Peer-reviewed journals from Web-of-Science

In the past evaluation period (2015-2019) we have been oriented to analytical and clinical mass spectrometry and achieved excellent results documented by 30 papers published in Q1 journals, see the snapshot below (six D1 journals are highlighted). Most of the original work from the lab, which performs its own and exciting research in the interrogated fields of microbiology, analytical chemistry, spectroscopy, imaging, medicine. Independency of the team is documented by several big grants, in which lab members have acted as PIs or co-PIs. Essential remained the support and services provided to collaborating academic and industrial partners, reflected by joint peer-reviewed and/or patent applications. The inventions were related to biotechnology processes in which microbial strains were used as whole-cell catalysts for bioremediation of persistent environmental pollutants from surface or drinking water sources.

2. Patents

- Volny, M., Novak, P., Havlicek, V., Pompach, P., Method of surface modification for analyzing phosphorylated peptides, Patent Number: US 09358573, Patent Assignee: Institute of Microbiology AS CR (2015).
- Microorganism *Raoultella* sp. KDF8 CCM 8678 degrading active pharmaceutical ingredients from the following group: diclofenac, codeine, ibuprofen, and ketoprofen. CZ307082-B6.
- The strain of the biodegradation of sulfonamide-based active compounds e.g. sulfathiazole in sewage or surface water involves adding bacterial strains mixture to wastewater as a metabolically active mixed culture of *Acinetobacter* species and *Kocuria* species. CZ307652-B6.

Laboratory of Molecular Structure Characterization

	Papers 2015	IF_{2015}	Quartile	$JCR^{®}$ Category	$JCR^{®}$AIS
1.	**Anal Bioanal Chem**; Krasny et al., 2015	3.125	Q1	CHEMISTRY, ANALYTICAL	0.823
2.	**Anal Bioanal Chem**; Kuchar et al., 2015	3.125	Q1	CHEMISTRY, ANALYTICAL	0.823
3.	**J Am Soc Mass Spectrom**; Novák et al., 2015	3.031	Q1	CHEMISTRY, ANALYTICAL	0.863
4.	**J Chromatogr A**; Pauk et al., 2015	3.926	Q1	BIOCHEMICAL RESEARCH METHODS	0.813
5.	**Mass Spectrom Rev**; Pluháček; Lemr, et al., 2015	9.346	Q1, D1	SPECTROSCOPY	2.234
6.	**Anal Bioanal Chem**; Pelantová et al., 2015	3.125	Q1	CHEMISTRY, ANALYTICAL	0.823
	Papers 2016	IF_{2016}	Quartile	$JCR^{®}$ Category	$JCR^{®}$AIS
7.	**The Analyst**; Hartmanová et al., 2016	3.885	Q1	CHEMISTRY, ANALYTICAL	0.795
8.	**PROTEOMICS**; Pluhacek et al., 2016	4.041	Q1	BIOCHEMICAL RESEARCH METHODS	1.113
9.	**Anal Chem**; Přichystal et al., 2016	6.320	Q1 (D1)	CHEMISTRY, ANALYTICAL	1.403
10.	**Bioconjugate Chem**; Zarska; Novotny, et al., 2016	4.818	Q1	BIOCHEMISTRY & MOLECULAR BIOLOGY	1.226
	Papers 2017	IF_{2017}	Quartile	$JCR^{®}$ Category	$JCR^{®}$AIS
11.	**Anal Chim Acta**; Borovcová et al., 2017	5.123	Q1	CHEMISTRY, ANALYTICAL	0.990
12.	**J Chromatogr A**; Kurka et al., 2017	3.716	Q1	BIOCHEMICAL RESEARCH METHODS	0.691
13.	**Sci Rep**; Luptáková et al., 2017	4.122	Q1	MULTIDISCIPLINARY SCIENCES	1.356
14.	**Anal Chim Acta**; Skultety et al., 2017	5.123	Q1	CHEMISTRY, ANALYTICAL	0.990
15.	**PLoS Genetics**; Kasparek et al., 2017	5.540	Q1	GENETICS & HEREDITY	3.329
16.	**J Med Chem**; Seydlová et al., 2017	6.253	Q1 (D1)	CHEMISTRY, MEDICINAL	1.495
17.	**J Chromatogr A**; Svobodová et al., 2017	3.716	Q1	CHEMISTRY, ANALYTICAL	0.691
18.	**J Endocrinol**; Bugáňová et al., 2017	4.012	Q1	ENDOCRINOLOGY & METABOLISM	1.231
	Papers 2018	IF_{2018}	Quartile	$JCR^{®}$ Category	$JCR^{®}$AIS
19.	**Sci Rep**; Luptakova et al., 2018	4.011	Q1	MULTIDISCIPLINARY SCIENCES	1.286
20.	**Sci Rep**; Petrik et al., 2018	4.011	Q1	MULTIDISCIPLINARY SCIENCES	1.286
21.	**Anal Chim Acta**; Pluháček; Švidrnoch, et al., 2018	5.256	Q1	CHEMISTRY, ANALYTICAL	0.994
22.	**Med Mycol**; Ramirez-Garcia et al., 2018	2.851	Q1, D1	VETERINARY SCIENCES	0.778
23.	**Front Microbiol**; Skriba et al., 2018	4.259	Q1	MICROBIOLOGY	1.254
24.	**J Hepatol**; Jirouskova et al., 2018	18.946	Q1 (D1)	GASTROENTEROLOGY & HEPATOLOGY	4.505
25.	**Biomaterials**; Zarska; Sramek, et al., 2018	10.273	Q1 (D1)	MATERIALS SCIENCE, BIOMATERIALS	1.989
	Papers 2019	IF_{2018}	Quartile	$JCR^{®}$ Category	$JCR^{®}$AIS
26.	**Front Microbiol**; Klimentova et al., 2019	4.259	Q1	MICROBIOLOGY	1.254
27.	**ALTEX**; Petrovova et al., 2019	6.183	Q1 (D1)	MEDICINE, RESEARCH & EXPERIMENTAL	1.040
28.	**Mat Sci Eng C-Mater**; Ryšánek et al., 2019	4.959	Q1	MATERIALS SCIENCE, BIOMATERIALS	0.751
29.	**J Proteome Res**; Čermáková et al., 2019	3.780	Q1	BIOCHEMICAL RESEARCH METHODS	1.161
30.	**Int Biodeterior Biodegrad**; Palyzová et al., 2019	3.824	Q1	BIOTECHNOLOGY & APPLIED MICROBIOLOGY	0.614

3. Grant resources

The financial independence is documented by the following major grants (selection):

- Ministry of Education, Youth and Sports of the Czech Republic (2015-2020, LO1509), National Sustainability Program – Prague Infrastructure for Structural Biology and Metabolomics II, 816 k€/duration, investigator
- Ministry of Education, Youth, and Sports of the Czech Republic, Danomics, 8X17052 (2017 – 2018), 12 k€/year, co-investigator
- Czech Science Foundation, Multimodal Imaging (2016-2018, 16-20229S), 261 k€/duration; investigator.
- Ministry of Education, Youth, and Sports of the Czech Republic, Analytical tools for fast identification of new designer drugs (2014-2016, Contact LH14064; 36 k€/duration; co-investigator, cooperation with University of Texas at Arlington, USA).
- Czech Science Foundation, Sampling and efficiency of atmospheric pressure desorption/ionization in mass spectrometric experiment (2012-2016, P206/12/1150, 134 k€/duration; co-investigator).
- European Cooperation in Science and Technology, grant No. BM1104 (2011-2015) and Ministry of Education, Youth and Sports of the Czech Republic (LD130038): Mass Spectrometry Imaging, 49 k€/duration, principal investigator

- Technology Agency of the Czech Republic (2017-2020, grant No. TH02030337), EPSILON Program for Support of Applied Research and Experimental Development, 283 k€/duration, co-investigator)

Research activity and characterisation of the main scientific results

2016 (the year when the lab was formally established)

Bird, J. G., Zhang, Y, Tian, Y, Panova, N, Barvík, I, Greene, L, Liu, M, Buckley, B, Krásný, L, Lee, J. K, Kaplan, C.D., Ebright, R.E., Nickels, B. E. (2016) The mechanism of RNA 5' capping with NAD+, NADH, and desphospho-CoA. **Nature**, 535(7612):444-447.

Chen and coworkers published surprising findings (Chen et al., 2009, Nature Chem. Biol. 5, 879–881), identifying NAD and dephospho-CoA as part of RNA in bacteria. Subsequently, Hana Cahova developed a chemical method that allowed to pull out and sequence the NAD-modified RNAs in E. coli (Cahova et al., 2015, Nature 519:374-377.). During a discussion with her I asked by what mechanism NAD was attached to RNA. The reply was that nobody knew at the time. People had tried to use RNAP and failed (Jaschke et al., 2016, Curr Opin Microbiol 30, 44-49). Nevertheless, considering the structure of NAD – the adenine nucleotide part is perfectly capable of base-pairing with a T in the template strand, and the 3' OH end of the ribose permits attachment of another nucleotide – I proposed that RNAP was still the likeliest enzyme responsible for this RNA modification. We used a different approach, however, than the earlier unsuccessful attempts. We used a radio-labeled NAD in a defined cell-free in vitro transcription system. The very first experiment then yielded a positive result: NAD utilization as the transcription initiating NTP was proven! Then, at the Phage Meeting in Madison, WI, USA, I met Jeremy Bird from the lab of Bryce Nickels who gave a talk describing essentially the same results. After that, we got into contact with Bryce and put our results together. Importantly, the Nickels lab also had a 3D crystallographic model of the RNAP complex with NAD that corroborated the whole idea and confirmed also our in silico modeling. In vivo, then, the presence of the cap (NAD) stabilized RNA against degradation, prolonging its biological half-life.

Contribution: 30 % (two other labs independently co-discovered the same phenomenon, resulting in this joint publication).

Raindlová, V., Janoušková, M., Slavíčková, M., Perlíková, P., Boháčová, S., Milisavljevič, N., Šanderová, H., Benda, M., Barvík, I., Krásný, L. and Hocek, M. (2016) Influence of major-groove chemical modifications of DNA on transcription by bacterial RNA polymerases. **Nucleic Acids Res**, 44(7):3000-12.

DNA templates containing a set of base modifications in the major groove (5-substituted pyrimidines or 7-substituted 7-deazapurines bearing groups with increasing bulkiness) were prepared by PCR using the corresponding base-modified 2'-deoxyribonucleoside triphosphates (dNTPs). The modified templates were used in an in vitro transcription assay using RNA polymerase from Bacillus subtilis and Escherichia coli. Some modified nucleobases bearing smaller modifications (H, Me in 7 deazapurines) were perfectly tolerated by both enzymes, whereas bulky modifications (Ph at any nucleobase) and, surprisingly, uracil blocked transcription.

Some middle-sized modifications (vinyl or ethynyl) were partly tolerated mostly by the E. coli enzyme. In all cases where the transcription proceeded, full length RNA product with correct sequence was obtained indicating that the modifications of the template are non-mutagenic and the inhibition is probably at the stage of initiation. The results laid the foundation for the subsequent development of bioorthogonal reactions for artificial chemical switching of the transcription.

Contribution: 50 %, we performed all the molecular biology experiments.

2017

Barvík, I., Rejman, D., Panova, N., Šanderová, H., Krásný, L. (2017) Non-canonical transcription initiation: The expanding universe of transcription initiating substrates **FEMS Microbiol Rev** 41(2): 131-8.

In this review we summarized the current knowledge about transcription initiation with a specific focus on various initiating substrates, including iNTPs, nanoRNAs (oligoribonucleotides), and coenzymes, such as NAD. Moreover, we predicted here that dinucleoside polyphosphates might function as transcription initiating substrates, a predictions that was subsequently confirmed (Luciano et al., 2019, Mol Cell.75(5):957-966.e8 and see also our 2020 Nat Comm paper).

Contribution: 85 %

Zachrdla M, Padrta P, Rabatinová A, Šanderová H, Barvík I, Krásný L, Žídek L (2017)Solution Structure of Domain 1.1 of the σA Factor from Bacillus subtilis is Preformed for Binding to the RNA Polymerase Core. **J Biol Chem** 292(28): 11640-17.

Sigma factors are essential for transcription initiation in bacteria. Domain 1.1 of primary sigma factors is relatively poorly characterized and we performed a structural study of this domain from SigA from Bacillus subtilis. The results revealed that it is highly compact because of additional stabilization not present in1.1 from the other two species (E. coli, T. maritima) for which their structures had been solved. This study helped understand how this domain might interact with the primary channel of RNAP.

Contribution: 50 %, we conceived the project, purified the protein fragment, our collaborators performed the NMR analysis.

Ramaniuk O, Černý M, Krásný L, Vohradský J (2017) Kinetic modelling and meta-analysis of the B. subtilis SigA regulatory network during spore germination and outgrowth. **Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms** 1860(8):894-904.

Here we defined the regulon of SigA during spore germination and outgrowth in Bacillus subtilis. We identified new SigA-dependent genes and experimentally validated the predictions.

Contribution: 40 %, we performed all the experimental validations (the first author is from my lab); the project was conceived and the computations were done by Jiri Vohradsky, a collaborator from our Institute.

Seydlová G, Pohl R, Zborníková E, Ehn M, Simak O, Panova N, Kolar M, Bogdanova K, Vecerova R, Fiser R, Šanderová H, Vítovská D, Sudzinová P, Pospíšil J, Benada O, Křížek T, Sedlak D, Bartunek P, Krasny L, Rejman D. (2017) Lipophosphonoxins II: Design, Synthesis and Properties of Novel Broad Spectrum Antibacterial Agents. **J Med Chem.** 60(14):6098-6118.

In this interdisciplinary study we describe the development and characterize novel antibacterial compounds, lipophosphonoxins II (LPPOs II). Relative to LPPOs I, LPPOs II display increased activity range, both against G+ and G- pathogens while not being cytotoxic. They function by permeabilizing the plasma mebrane.

Contribution: 30 %, we performed all the mechanistic studies involving live cells, organic chemists designed and synthesized the compounds, G. Seydlova performed in vitro experiments with membranes.

Srb, P., Nováček, J., Kadeřávek, P., Rabatinová, A., Krásný, L., Žídková, J., Bobálová, J., Sklenář, V., Žídek, L. (2017) Triple resonance 15N NMR relaxation experiments for studies of intrinsically disordered proteins. **J. Biomol. NMR.** 69(3):133-146.

A methodical study describing an NMR method.

Contribution: 10 %, we designed and purified the proteins.

Janoušková, M., Vaníková, Z., Nici, F., Boháčová, S., Vítovská, D., Šanderová, H., Hocek, M., and Krásný, L. (2017) 5-(Hydroxymethyl)uracil and -cytosine as potential epigenetic marks enhancing or inhibiting transcription with bacterial RNA polymerase. **Chem Comm** 53(99):13253-13255.

In this study we used several bacterial promoters and RNAP from E. coli to investigate the effect of 5-methylcytosine (5mC) and 5-hydroxymethyluracil (5hmU) on transcription. We showed that both 5hmC
and 5hmU affect transcription by bacterial RNAP depending on the promoter sequence. The effects were both inhibitory and, surprisingly, also stimulatory. This study was the first to report strong enhancement of transcription of templates containing 5hmU or 5hmC in an in vitro enzymatic assay. We then focused on 5hmU where the effects were more pronounced. In the case of 5hmU, both the enhancement and inhibition were mediated predominantly by interactions of the promoter non-template strand with RNAP. We note that while we used for our studies a well-characterized promoter (Pveg), other promoters may exist in the genome where random modifications of even single bases may have even more pronounced effects on transcription. Taken together, this illustrates the strong potential of 5hmU to alter gene expression in vivo.

Contribution: 50 %, we performed all the molecular biology experiments.

2018

Slavíčková, M., Janoušková, M., Šimonová, A., Cahová, H., Kambová, M., Šanderová, H., Krásný, L., Hocek, M. (2018) Turning off transcription with bacterial RNA polymerase through CuAAC clisk reactions of DNA containing 5-ethylenuracil. **Chemistry - A European Journal** 24(33):8311-8314.

An in vitro study where we demonstrated the possibility of turning bacterial transcription OFF with biorthogonal biology chemistry.

Contribution: 50 %, we performed all the molecular biology experiments.

Vvedenskaya, I. O., Bird, J. G., Zhang, Y, Zhang, Y, Jiao, X., Barvík, I., Krásný, L., Kiledjian, M., Taylor, D. M., Ebright, R. H., Nickels, B. E. (2018) "CapZyme-Seq" comprehensively defines promoter-sequence determinants for RNA 5' capping with NAD+. **Mol Cell** 3;70(3):553-564.

We characterised the promoter sequence determinants of RNA-capping by NAD.

Contribution: 10 %, we performed in silico modelling and characterisation of an initial set of modified promoters.

Ramaniuk O, Převorovský M, Pospíšil J, Vítovská D, Kofroňová O, Benada O, Schwarz M, Šanderová H, Hnilicová J, Krásný L. (2018) sI from Bacillus subtilis: Impact on gene expression and characterization of sI-dependent transcription that requires new types of promoters with extended -35 and -10 elements. **J. Bacteriol** pii: e00251-18. doi: 10.1128/JB.00251-18.

SigI was the least explored/defined sigma factor regulon in B. subtilis at the beginning of our studies. Considering the industrial importance of this model organism, we decided to define its regulon and cellular roles. SigI had been previously implicated in adaptation of the cell to elevated temperature. In this study, we provided a comprehensive characterization of this transcriptional regulator.

Contribution: 85 %, collaborators performed RNAseq analysis and electron microscopy.

Sykora, M., Pospisek, M., Novak, J., Mrvova, S., Krásný, L., Vopalensky, V. (2018) Transcription apparatus of the yeast virus-like elements: Architecture, function, and evolutonary origin. **PLoS Pathog**. 14(10):e1007377.

We described the architecture and identified the evolutionary origin of the transcription machinery of linear plasmids (also known as virus-like elements, VLS) from Kluyveromyces lactis. We showed that the two RNAP subunits interact in vivo, and this complex interacts with another two VLE-encoded proteins, namely the mRNA capping enzyme and a putative helicase. RNAP, mRNA capping enzyme and the helicase also interact with VLE-specific DNA in vivo. Further, we identified a promoter sequence element that causes 5' mRNA polyadenylation of VLE-specific transcripts via RNAP slippage at the transcription initiation site, and structural elements that precede the termination sites. As a result, we presented a first model of the yeast virus-like element

transcription initiation and intrinsic termination. Finally, we demonstrated that VLE RNAP and its promoters display high similarity to poxviral RNAP and promoters of early poxviral genes, respectively, thereby pointing to their evolutionary origin.

Contribution: 15 %, I contributed to the project design and wrote the manuscript.

2019

Kouba T, Pospíšil J, Hnilicová J, Šanderová H, Barvík I, Krásný L. (2019) The core and holoenzyme forms of RNA polymerase from Mycobacterium smegmatis. **J. Bacteriol** 201(4). pii: e00583-18. doi: 10.1128/JB.00583-18.

We solved the cryo-EM structure of two forms of RNAP from Mycobacterium smegmatis.This is now a foundation for complexes of this RNAP with other factores (work in progress).

Contribution: 50 %, we conceived the project, we created an expression system for Msm RNAP in E. coli, purified the enzyme, performed transcription activity experiments with RNAP, participated in interpretation of the data; T, Kouba solved the structure.

Schäfer H, Heinz A, Sudzinová P, Voß M, Hantke I, Krásný L, Turgay K. (2019) Spx, the central regulator of the heat and oxidative stress response in B. subtilis can repress transcription of translation-related genes. **Mol Microbiol**. 111(2):514-533.

This study characterizes the role of Spx in heat and oxidative stress. It also contains detailed in vitro study validating the observed effects identified by large scale approaches (e. g. ChIPseq).

Contribution: 15 %, we performed in vitro transcriptions, participated in experimental design.

Šiková M, Janoušková M, Ramaniuk O, Páleníková P, Pospíšil J, Bartl P, Suder A, Pajer P, Kubičková P, Pavliš O, Hradilová M, Vítovská D, Šanderová H, Převorovský M, Hnilicová J, Krásný L. (2019) Ms1 increases the amount of RNA polymerase in Mycobacterium smegmatis. **Mol Microbiol**. 111(2):354-372.

Here we characterized the effects of the sRNA, Ms1, on RNAP in M. smegmatis. We showed that it sequesters the RNAP core during stationary phase of growth, and the absence of Msq results in slower outgrowth. Furthermore, Ms1 affect expression of RNAP by an unknown mechanism.

Contribution: 80 %, our collaborators performed RNAseq analysis, electron microscopy, and radiation-resistance phenotypic experiments.

Vaníková, Z, Martina Janoušková, M., Kambová, M., Krásný and Hocek, M. (2019) Switching transcription with bacterial RNA polymerase through photocaging, photorelease and phosphorylation reactions in the major groove of DNA. **Chemical Science**, 10(14):3937-3942.

The study describes proof of principle switching of transcription in vitro through non-natural chemical reactions in the major groove of DNA templates. Photocaged DNA templates containing bulky modifications permitted no transcription with E. coli RNAP (OFF state). Their irradiation with 400 nm light resulted in DNA templates containing small modifications, switching transcription ON Phosphorylation of templates containing these small modifications then turned transcription OFF again.

Contribution: 50 %, we performed all the molecular biology experiments.

Kovaľ, T., Sudzinová, P., Perháčová, T., Trundová, M., Skálová, T., Fejfarová, K., Šanderová, H., Krásný, L., Dušková, J., Dohnálek, J. (2019) Domain structure of HelD – an RNAP interacting partner from Bacillus subtilis. **FEBS Letters** 593(9):996-1005. doi: 10.1002/1873-3468.13385.

We characterized the overall shape of HelD from B. subtilis and the functional aspects of its N-terminal domain. HelD is a binding partner of RNAP, previously discovered in our laboratory (Wiedermannova et al., 2014, Nucleic Acids Res 42(8): 5151-63.) that participates in recycling of RNAP during transcription termination. This study paved the way to the current structure of RNAP with HelD (manuscript in preparation).

Contribution: 30%, we performed the functional studies, participated in results interpretation.

Zborníková E, Knejzlík Z, Hauryliuk V, Krásný L, Rejman D. (2019) Analysis of nucleotide pools in bacteria using HPLC-MS in HILIC mode**. Talanta.** 205:120161.

A new and facile method to analyse nucleotide pools from bacterial cells.

Contribution: 15 %, I participated in the design and co-wrote the manuscript.

Kubáň V, Srb P, Štégnerová H, Padrta P, Zachrdla M, Jaseňáková Z, Šanderová H, Vítovská D, Krásný L, Koval' T, Dohnálek J, Ziemska-Legiecka J, Grynberg M, Jarnot P, Gruca A, Jensen MR, Blackledge M, Žídek L. (2019) Quantitative Conformational Analysis of Functionally Important Electrostatic Interactions in the Intrinsically Disordered Region of Delta Subunit of Bacterial RNA Polymerase. **J Am Chem Soc.** 141(42):16817-16828.

Intrinsically disordered proteins are common in cells. Their flexibility is key for their respective functions. Here, we comprehensively characterized the unstructured part of the delta subunit and described the role of the lysine-rich tract preceding this unstructured part. The tract restricts the movements of the flexible acidic part and is essential for its proper function. By an array of experiments we demonstrated that it is central to physiologically correct transcription initiation and the survival of the cell.

Contribution: 50 %, we conceived the project, performed all biochemical and phenotypic experiments. Our collaborators performed the extensive structural characterisation of the interactions between the lysine tract and the unstructured domain.

LIPOPHOSPHONOXINS OF SECOND GENERATION, AND THEIR USE
European etc. patents
AU2017257061A1;AU2017257061B2;CA3021537A1;CZ2016243A3;WO2017186200A1;EP3448865A1;EP3448865B1

Contribution: 10 % (share on the patents)

2020

(These results are not subject to the evaluation as the papers were published this year but the bulk of the work was done in previous years, so I mention these papers here for the completeness sake.)

Šiková, M., Wiedermannová, J., Převorovský, M., Barvík, I., Sudzinová, P., Kofroňová, O., Benada, O., Šanderová, H., Condon, C., Krásný, L. (2020) The Torpedo Effect in Bacillus subtilis: RNase J1 Resolves Stalled Transcription Complexes. **EMBO J** 39(3):e102500. doi: 10.15252/embj.2019102500.

We describe here a novel mechanism – a 5' to 3' exonuclease, RNAse J1 from Bacillus subtilis is able to dissociate (to "torpedo") stalled RNAP complexes, thereby preventing potentially deleterious transcription-replication clashes.

Contribution: 90 %, we designed the study, performed the majority of the experiments. Our collaborators performed –omic analyses, electron microscopy and in silico modelling.

Zborníková, E.,Gallo, J., Večeřová, R.,; Bogdanova, K., Kolář, M., Vítovská, D., Do Pham, D. D., Paces, O., Mojr, V., Šanderová, H., Ulrichová, J., Galandáková, A., Čadek, D., Hrdlička, Z., Krásný, L., Rejman, D. (2020) Evaluation of 2nd generation lipophosphonoxins as antimicrobial additives in bone cement. **ACS Omega** doi.org/10.1021/acsomega.9b03072 (in press)

In this study we showed an excellent potential of LPPOs II as additives to surgical cements.

Contribution: 15 %, a large collaboration study, we co-designed the study, performed elution kinetics experiments, participated in results analysis, and co-wrote the manusctipt.

Hudeček, O., Benoni, R., Reyes-Gutierrez, P. E., Culka, M., Šanderová, H., Hubálek, M., Rulíšek, L., Cvačka, J., Krásný, L., Cahová, H. (2020) Dinucleoside polyphosphates act as 5'-RNA caps in bacteria. **Nat. Comm.** (in press)

We demonstrated that dinucleoside polyphosphates can be used by E. coli RNAP as transcription initiating substrates. Moreover, we detected these molecules covalently attached to 5' of RNAs in E. coli, true prokaryotic caps. Finally, we showed that ApaH participates in removal of these caps from RNA.

Contribution: 20 %, we conceived the original idea (see our review from 2017), purified ApaH, co-wrote the manuscript.

Research activity and characterisation of the main scientific results

Scientific activities of the Laboratory team can be divided into the following specific areas:

1) **Corynebacterium: Construction of a model of regulatory network controlled by sigma factors of RNA polymerase in *Corynebacterium glutamicum***
This project represents the long-term direction of the studies in the Laboratory. *Corynebacterium glutamicum* is a biotechnological bacterium used for the production of amino acids and other metabolites. We concentrated on the studies of the roles of sigma factors of RNA polymerase in the regulatory mechanisms of transcription. The main points included into these studies were stress response in *C. glutamicum*, sigma regulons, partially overlapping regulons, promoter classes and promoter consensus sequences.
The project has been focused on elucidating the roles of the alternative sigma factors of RNA polymerase in expression of *Corynebacterium glutamicum* genes and on integrating all available data into a model of sigma factor regulatory network. We developed the unique in vitro transcription system for *C. glutamicum* and constructed two-plasmid system for in vivo assignment of sigma factors to particular promoters. We used the reconstituted holo-RNAP for in vitro assays of individual promoters. Moreover, in silico modeling of recognition of the key promoter nucleotides by RNAP+sigma proteins contributed to a complex view on the relations of individual holo-RNAPs to different classes of promoters. The results of RNA sequencing produced by collaborating group at Bielefeld University provided us analysis of transcriptome generated in the presence of particular sigma factors or under specific stresses (e.g. phenol stress). We combined these genome-level results with our single gene/promoter results (in vivo, in vitro and in silico). On the basis of this combination of techniques, reliable description of sigma regulons, classes of promoters and their promoter consensus sequences were obtained. A model of sigma factor regulatory network has been proposed. The grant project was completed in 2019 and large amount of the obtained final results will be published in 2020.

Pahlke J., Dostálová H., Holátko J., Degner U., Bott M., Pátek M., Polen T., Small 6C RNA of *Corynebacterium glutamicum* is involved in DNA damage response. RNA Biology 13:848-860 (2016)

Three co-authors (HD, JH and MP) out of 7 are the members of the Lab team. Their contribution to the paper can be estimated to 40%. Our team worked on transcriptional regulation of 6C RNA synthesis and effects of DNA-damaging compounds on 6C RNA level.

Šilar R., Holátko J., Rucká L., Rapoport A., Dostálová H., Kadeřábková P., Nešvera J., Pátek M.: Use of in vitro transcription system for analysis of *Corynebacterium glutamicum* promoters recognized by two sigma factors. Curr Microbiol 73:401–408 (2016).
The paper was completely produced by the Lab team (100%).

Dostálová H., Holátko J., Busche T., Rapoport A., Rucká L., Halada P., Nešvera J., Kalinowski J., Pátek M.: Assignment of sigma factors of RNA polymerase to promoters in *Corynebacterium glutamicum* (2017) AMB Express 7:133 (2017).
Most of the experiments were done by our Lab team (HD, JH, AR, JN, MP). We used RNA-seq data provided by the colleagues from Bielefeld University. A colleague from the Institute of Microbiology (PH) contributed by results of mass spectrometry.

Taniguchi H., Busche T., Patschkowski T., Niehaus K., Pátek M., Kalinowski J. Wendisch J.F. Physiological roles of sigma factor SigD in *Corynebacterium glutamicum* BMC Microbiology 17:158 (2017)
A single co-author (MP) is a member of the Lab. His contribution by the idea and writing the paper can be estimated to 10%.

Dostálová H., Busche T., Holátko J., Rucká L., Štěpánek V., Barvík I., Nešvera J., Kalinowski J., Pátek M.: Overlap of promoter recognition specificity of extracytoplasmic function sigma factors SigD and SigH in Corynebacterium glutamicum ATCC 13032 Frontiers in Microbiol 9:3287 (2019).
Transcriptome sequencing was done by the colleagues from Bielefeld University. We did all other experimental work and completed the manuscript (HD, JH, LR, VŠ, JN, MP). A colleague from Institute of Physics (IB) carried out the in silico modeling.

2) **Rhodococcus: Stress responses in bacterial degrader of toxic pollutants *Rhodococcus erythropolis***
Strains of the bacterial genus *Rhodococcus*, classified into nocardioform actinomycetes, are very efficient in biodegradation of various toxic compounds, which contaminate environment. The presence of toxic contaminants and the environmental conditions at the polluted sites exert stress on the cells due to high concentrations of toxic compounds, solvents, extreme pH, temperature and osmolarity. We first analyzed the data of phenol stress response in *Rhodococcus erythropolis* (phenol was used as a model pollutant). Using RNA sequencing we compared transcriptomes of *R. erythropolis* grown on glycerol and phenol, respectively. Phenol stress resulted in hundreds of differentially expressed gene. We detected 400 up-regulated genes and analyzed 30 of them, which were regulated by stress sigma factors. We found genes controlled by SigB, SigD, SigE, SigH and SigK. In addition to phenol stress, we analyzed the SigE and SigH regulons using RNA sequencing. We have thus obtained complementary data about 86 up-regulated stress genes (35 by SigE and 51 by SigH) controlled by two main sigma factors in *R. erythropolis*.
On the basis of the analysis of sigma factor dependence of the *sig* genes, we have constructed the core regulatory network of *R. erythropolis.*
This is the first detailed and complex research of the stress response in *Rhodococcus* strains on the level of transcription and of the role of alternative sigma factors in these processes. The data obtained by this analysis of the particular genes involved in stress response will serve as a basis for explaining the mechanisms of resistance to stress factors and efficient degradation of toxic environmental contaminants. The data sets integrated into regulatory models can also be transferred to related *Rhodococcus* strains (e.g. *R. jostii* RHA1) by comparative genomics and appropriate bioinformatic tools.

Nešvera J., Rucká L., Pátek M.: Catabolism of phenol and its derivatives in bacteria: genes, their regulation and use in the biodegradation of toxic pollutants Adv. Appl. Microbiol. 93:107-160 (2015)
Rucká L., Nešvera J., Pátek M.: Biodegradation of phenol and its derivatives by engineered bacteria: current knowledge and perspectives. World J. Microbiol. Biotechnol. 33: 174 (2017)

Both overviews of the phenol stress and catabolism were produced exclusively by the members of the Lab team.
The grant project is at the third year in 2020. Large amount of results serve currently as a basis for publications.

3) **Nitrile:** Studies of the enzymes of nitrile metabolism
We collaborate with L. Martínková (Institute of Microbiology) in this topic

New aldoxime dehydratases: isolation, characterization and use in biocatalytic modules. (L. Rucká, R. Rädisch, H. Dostálová, M. Pátek)

Aldoxime dehydratases (AODases) are emerging biocatalysts, which have not yet been applied adequately in organic chemistry. The main aim of this project is to discover new activities of potential AODases, which are so far only known as sequences in databases, and create artificial catalytic biomoduls of AODases with nitrilases, nitrile hydratases and amidases.
We detected the supposed AODases by data mining in the sequenced genomes of fungi and plant-related bacteria.
We synthesized several genes for aldoxime dehydratases and proved activities for 4 purified recombinant enzymes. These enzymes encoded by the *oxd* genes originated in *Bradyrhizobium* sp. (2 proteins) , *Pseudomonas* sp. and *Trichoderma virens*. The enzyme OxdBr1 from *Bradyrhizobium* sp. is active with (aryl)aliphatic aldoximes and is highly homologous with aldoxime dehydratases that are present in plant-symbiotic members of the *Bradyrhizobium* genus, where they can interfere with auxin production or defense mechanisms in the host plant. According to analysis of the genes in the operon, OxdBr1appears to be part of the aldoxime-nitrile pathway in *Bradyrhizobium* sp. The transforming potential of OxdBr1 is significant and the enzyme can be used without using anaerobic conditions in contrast to some previously reported AODases.

New "nitrilase superfamily" proteins in Basidiomycota: study of their activities and potential functions in the biodegradation of cyanide and nitriles
(L. Rucká, R. Rädisch, H. Dostálová, M. Pátek)

We found more than 200 putative nitrilases from Basidiomycota in GenBank. We analyzed their sequences and classified them into phylogenetic clades. Representatives of clade 1 and 2 (NitTv1 from *Trametes versicolor* and NitAg from *Armillaria gallica*, respectively) and a putative CynH (NitSh from *Stereum hirsutum*) were overproduced in *Escherichia coli*. The substrates of NitTv1 were (aryl)aliphatic aldoximes and beta-cyano-l-alanine and 4-cyanopyridine, and those of NitSh were hydrogen cyanide (HCN), 2-cyanopyridine, fumaronitrile and benzonitrile. NitAg only exhibited activities for HCN and fumaronitrile. The substrate specificities of these nitrilases were largely in accordance with substrate docking in their homology

models. Nitrilases in Basidiomycota need to be studied further with focus on the regulation of their production in fungi. This should help to better understand and exploit the bioremediation potential of these fungi.

To continue this research area we have started a new Czech-Austrian grant project **A new chemoenzymatic route from carboxylic acids to nitriles.**
(L. Rucká, R. Rädisch, M. Pátek)

Veselá A. B., Rucká L. Kaplan O., Pelantová H., Nešvera J., Pátek M., Martínková L. Bringing nitrilase sequences from databases to life: the search for novel substrate specificities with a focus on dinitriles Appl Microbiol Biotechnol 100:2193-2202 (2016)

Martínková L., Rucká L., Nešvera J., Pátek M. Recent advances and challenges in the heterologous production of microbial nitrilases for biocatalytic applications. World J. Microbiol. Biotechnol. 33:8 (2017)

Rädisch R., Chmátal M., Rucká L., Novotný P., Petrásková L., Halada P., Kotik M., Pátek M., Martínková L.: Overproduction and characterization of the first enzyme of a new aldoxime dehydratase family in Bradyrhizobium sp. Int. J. Biol. Macromol. 115:746-753 (2018)

Rucká L., Chmátal M., Kulik N., Petrásková L., Pelantová H., Novotný P., Příhodová R., Pátek M., Martínková L.: Genetic and functional diversity of nitrilases in *Agaricomycotina* Int. J. Mol. Sci. 20:5990 (2019)

L. Rucká, R. Rädisch, J. Nešvera and M. Pátek contributed substantially at all stages of the studies: Conceiving the study, gene cloning, enzyme isolation and purification, enzyme activity measurements and manuscript preparation and editing.

4) **Metagenome: Metagenomic organization of genes with functional priority for degradation of aromatic hydrocarbons in highly contaminated environmen**t

Metagenomic studies of natural attenuation: The project is focused on biodegradation of persistent aromatic pollutants (petroleum hydrocarbons, polychlorinated biphenyls, and chloroethenes). During the past 5 years, the research concentrated on microbial communities and biodegradation genes in aquifers highly contaminated with jet fuel and polyaromatic hydrocarbons. Metagenomic libraries were assessed by functional screening for the presence of extradiol dioxygenase activity. High-throughput sequencing of positive metagenomic clones led to the identification of subfamily of orthologous genes with functional priority for the degradation of aromatic hydrocarbons in the highly contaminated environment. Their genomic organization, expression in the soil, or heterologic expression were studied.

Praveckova M., Brennerova M.V., Cvancarova M., De Alencastro L.F., Holliger C., Rossi P.: Divergent PCB organohalide-respiring consortia enriched from the efflux channel of a former Delor manufacturer in Eastern Europe. Ecotox Environ Safe 120: 223–234 (2015)

Praveckova M., Brennerova M.V., Holliger C., De Alencastro L.F., Holliger C., Rossi P.: Indirect evidence link PCB dehalogenation with Geobacteraceae in anaerobic sediment-free microcosms. Frontiers in Microbiol 7:933 (2016)

5) **Nanomedicine**: **Alterations and targeting of RNA molecules involved in human pathologies (diabetes, neurodegeneration, and cancer)**

The newly joined group of V. Benson focuses on the development of new therapeutical approaches using perspective nanomaterial specifically nanodiamond. The nanodiamond particles were found to be promising carriers of short RNAs. Developed methodology has been further improved for in vivo application in cancer model. Moreover, we performed in vivo study on model of healing diabetic ulcers leading to an applied output (patent application before submission). In parallel, the group studies regeneration of neurons on a nanocrystalline diamond film ex vivo. The film was used as coating of intracranial electrode in order to gain durability. In practice, it can be used for deep brain stimulation in patients with neurodegenerative diseases. (V. Benson, E. Neuhöferová + technician L. Rendlová)

Lukowski S., Neuhoferova E., Kinderman M., Krivohlava R., Mineva A., Petrakova V., Benson V.: Fluorescent nanodiamonds are efficient, easy-to-use cyto-compatible vehicles for monitored delivery of non-coding regulatory RNAs. J Biomed Nanotechnol 14:1–13 (2018).

Křivohlavá R., Neuhöferová E., Jakobsen K.Q., Benson V. Knockdown of microRNA-135b in mammary carcinoma by targeted nanodiamonds: potentials and pitfalls of in vivo applications. Nanomat 9:866-887 (2019)

Krůšek J, Dittert I., Smejkalová T., Kořínek M., Gottfriedová K., Freislebenová H., Neuhöferová E., Klimša L., Sedláková S., Taylor A., Mortet V., Petrák V., Benson V., Petráková V. Molecular functionalization of planar nanocrystalline and porous nanostructured diamond to form an interface with newborn and adult neurons. Phys Status Solidi (B) 256 (3):1800424-1800433 (2019)

6) **Restriction-modification systems**

Restriction-modification systems of Type I. were studied.
This fully functional enzymes restriction-modification enzymes of Type I. consists of a complex of the three subunits HsdR, HsdM and HsdS. Analysis of the assembly pathway of the enzyme EcoR124I was performed using three methods: electrophoretic mobility shift assay (EMSA), surface plasmon resonance (SPR) and atomic force microscopy (AFM).
The complementary techniques provided consistent results and inherent differences in the methodologies provide additional information useful for the study of subunit assembly.

Csefalvay E., Lapkouski M., Guzanova A., Csefalvay L., Baikova T., Shevelev I., Bialevich V., Shamayeva K., Janscak P., Kuta Smatanova I., Panjikar P., Carey J., Weiserova M., Ettrich R.: Functional coupling of duplex translocation to DNA cleavage in a Type I restriction enzyme. Plos One 10:e0128700 (2015)

Youell J., Sikora A. E., Vejsadová Š., Weiserová M., Smith J. R., Firman K.: Cofactor induced dissociation of the multifunctional multisubunit EcoR124I investigated using electromobility shift assays RSC Adv. 7:38737–38746 (2017)

Bialevich V., Sinha D., Shamayeva K., Guzanova A., Csefalvay E., Carey J., Weiserova M., Ettrich R.: The helical domain of the EcoR124I motor subunit participates in ATPase activity and dsDNA translocation. Peer J 5:e2887 (2017)

Three co-authors (AG, ŠV and MW) participating on this research were the members of the Lab team. The most important contributions to the papers were constructions of the mutants in various enzyme subunits and evaluation of their activities. The leader of the group, M. Weiserová, retired in 2018 and the program ended.

Research activity and characterisation of the main scientific results

The Laboratory focus have had three main parts – 1. sigma factors regulatory networks in bacillus and streptomyces, development of modelling and meta-analysis approaches, 2. RNA secondary structure analysis and prediction, 3. building of a bioinformatics infrastructure.

ad1. we focused on meta-analysis and computational modelling of sigma factors control networks. The idea consists in the utilization of a computational model of gene expression based on time series of gene expression processed by an ordinary differential equation model. The model predicts the kinetics of gene expression which is then compared with actual gene expression kinetics. The comparison allows identification of genes whose expression time series are coherent with the model and indicates that such regulation for the used time series is possible. These results are combined with the data obtained from large scale meta analyses coming either from genomic experiments and/or literature mining. The “static” methods as RNA-seq or ChIP-seq alone are not sufficient as they provide information about actual binding to a promoter region of a gene but not whether the interaction results in actual expression of the gene. The presented approach allows identification of the regulation which really takes place under the conditions reflected in the analysed time series. The model also allows to incorporate activity of other transcription factors. The model was implemented as an R package, a command line tool (available at GitHub), and was accepted by CYTOSCAPE platform as its plugin. The tool was published in Bioinformatics Journal, both authors were members of the lab. The model was combined with the information mined in literature for the case of B. subtilis,and with the binding information given by a ChIP-seq experiment in streptomyces. This approach lead to identification of active regulatory networks for principal sigma factors SigA and SigB (others ongoing) in bacillus and HrdB/RbpA complex, SigQ, SigE and sigR (ongoing) in streptomyces. Results were published in the BBA Gene Regulatory Mechanisms, NAR, BMC bioinformatics journals. The collaborating laboratories made experiments that confirmed predictions of the model, the corresponding author conceived the work, made all the computations and wrote the paper. Concomitantly, the model of gene expression control was applied for clarification of the observed behaviour of the phage lambda, which exhibit in the host cell exclusively one of its stages; lysis or lysogeny, which never overlap. The model explained this behaviour as a feature of its regulatory network. The computational simulation of gene expression over time showed that when the system settles in one of the stages, it never flips into an opposite stage even when the concentration of the control molecules changes hundred fold. The mathematical simulation shows that this feature resembles systems with critical behaviour known from other scientific disciplines. This was a theoretical single author paper published in the Journal of Theoretical Biology.

ad2. we focused on RNAs structural computational biology. In particular, on the prediction of new ncRNAs and identification of function of RNAs using computational structure modelling. The computational approaches were complemented with experimental verification made in the collaborating labs. Five projects have been running under this topic. 1. a bioinformatics prediction of the role of spliceosomal

RNAs (snRNAs) in splicing. We identified a previously unknown role of snRNAs that initializes formation of spliceosomes. We predicted and experimentally proved that metazoan pre-snRNAs of major spliceosomes most likely interact with Gemin3 and Gemin5, the proteins of the SMN complex with previously unknown function. The interaction causes structural rearrangement of pre-snRNAs that makes it possible to bind Sm-ring to pre-snRNAs, which leads to snRNPs assembly. Thus the structure rearrangement is the first, previously unknown step of the spliceosomal formation. The experimental work has been done in collaboration with the Lab. of RNA biology at the Inst. of Molecular Genetics. 2. a bioinformatic prediction of ms1 and 6S RNAs, small non-coding regulatory bacterial RNAs, in Actinobacteria. These two RNAs function most likely as transcriptional regulators in bacterial cells. Our computational search based on the RNA structure homology identified putative candidates for both ms1 and 6S RNAs in Actinobacteria. Previously it was thought that 6S RNA occurs only in G- bacteria, whereas ms1 only in Actinobacteria. We suggest that both may exist in Actinobacteria. The prediction is currently experimentally verified in collaborating Lab. of Microbial Genetics and Gene Expression at our Institute. 3. - 5. tools for secondary structure predictions implemented as www servers - cpPredictor, http://cppredictor.elixir-czech.cz/, a webserver for prediction of RNA secondary structures based on an algorithm for template-based RNA secondary structure prediction developed in the Lab. rPredictorDB, http://rpredictordb.elixir-czech.cz, a predictive database of secondary structures of individual RNAs predicted using an algorithm for template-based RNA secondary structure prediction and their formatted plots developped in the Lab in collaboration with Faculty of Mathematics and Physics of the CU, Prague.5. rboAnalyzer, https://github.com/cas-bioinf/rboAnalyzer, a tool for characterizing HSPs in output of RNA BLAST search. The algorithm and the webservers were published in Frontiers in Genetics, in Bioinformatics and Database journals.

ad3. the Laboratory focused on building of a bioinformatics infrastructure at the Institute under the framework of the large bioinformatics infrastructure ELIXIR. Under this project, partially financed by the ELIXIR with the support of the Institute, a bioinformatics core facility was established and currently employs two FTE. The core facility has participated in number of projects within the Institute and other academic institutions e.g. Inst. of Microbiology, Visual diagnostics for metagenomics data, analyzing proteomics data from patients with Bordetella Pertussis. Inst. of Molecular Genetics – Meta-analysis of genotype-phenotype relationship in Bardet-Biedl syndrome, Modelling of miRNA degradation in mammalian oocytes, . The University of Melbourne, Australia, Statistical modelling of RNA-seq data. Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro Comparing biorXiv preprints with articles from PubMed to investigate the effect of peer review empirically, 2nd Medical faculty CU, Prague, Statistical analysis of epigenetic datasets. Stan Development Team, Columbia University, New York; Aalto University, Helsinki (and elsewhere), development of the Stan probabilistic programming language and associated tools and learning materials.

We also participate in the tuition of bioinformatics for the Second Medical Faculty of the Charles University, Prague.

We are also involved, together with the Laboratory of the Environmental microbiology of the Institute, in the building of a Biodiversity Community within the ELIXIR

infrastructure. For the future, we consider the development of the infrastructure as the most important direction for the development of the Laboratory.

Research activity and characterisation of the main scientific results

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

We identified **new cell division protein**, named **LocZ** (previously **Spr0334** with unknown function) for "Localizing at midcell of FtsZ", 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 cell 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 that 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. These results were published in **Holečková et al (2015) mBIO 6(1), e01700-14 (IF2015 = 6.7, Times cited: 42)**. The article was recommended by Faculty of 1000Prime service and rated as „very good". Except for the electron microscopy (O. Benada, Group of electron microscopy of the Institute of Microbiology), all the experimental work was done in our laboratory. Our Italian collaborator (O. Massidda) contributed to the manuscript preparation. The team's contribution to the manuscript is 90 %.

Further, we investigated the function of **FtsA**, **actin-like protein**, in *S. pneumoniae*. We have characterized three *S. pneumoniae ftsA* thermosensitive (TS) mutants, obtained by error-prone random PCR, allelic replacement and subsequent screening for their ability to grow at 28°C (permissive temperature), performed the identification and characterization of S. pneumoniae ftsA conditional lethal mutants and showed that inactivation of ftsA is not tolerated, confirming that **FtsA** is **essential** in ***S. pneumoniae***. Partial depletion of FtsA inhibits septation, and more complete depletion causes isotropic cell expansion and lysis. In contrast to the model rods, **inactivation of SpFtsA** results in **cell lysis** rather than cell filamentation, suggesting that **both elongation and division are blocked** in its absence. Moreover, our data suggest a model in which **FtsA directs both peripheral and septal cell wall synthesis**, functionally replacing the actin-like rod-shape MreB determinant, that in has been likely lost during the transition from the rod to the ovococcal shape. Taken together, these results confirm that *ftsA* is an essential gene and validate FtsA as a cell division target in *S. pneumoniae*. Moreover, they suggest that FtsA is necessary

at the earliest stages of cell division and may act, together with FtsZ, in organizing both the cell division and the elongation complexes required to achieve the oval shape. These results were published in **Mura et al (2017) J. Bacteriol 199(3), e00608-16 (IF2017 = 3.2, Times cited: 14**). Recently, when **T404** residue was identified as a **site of phosphorylation**, we prepared recombinant FtsA T404A mutant protein and performed in vitro kinase assay. The obtained results suggest that there is another phosphorylated aa residue to be determined. Beside the early stage experiments (mutant preparation) all experimental work was almost entirely performed at Institute of Microbiology. Collaborating teams participated in result interpretation, manuscript writing and editing.

In collaboration with Winklers' lab we investigated the **function** of another **StkP substrate**, **regulatory protein GpsB**, which has been proposed as molecular switch that balance septal and peripheral peptidoglycan synthesis in ***S. pneumoniae***. In primary, wild type progenitor strains, such as virulent strains D39 and TIGR4 and isogenic unencapsulated (Δcps) derivatives of D39, **GpsB is essential** for growth. Depletion of GpsB in D39 Δcps strains causes cultures to stop growing and eventually to lyse. We demonstrated that the **loss of GpsB can be suppressed** by spontaneous mutations, including within the **gene encoding** the only **PP2C Ser/Thr phosphatase PhpP,** indicating that **GpsB plays a key** – but unknown – **role in protein phosphorylation in pneumococci**. Further, we determined that **mutant alleles of phpP encode enzymatically inactive variants of PhpP**. We confirmed that reduction of GpsB amount leads to decreased protein phosphorylation by StkP. Therefore, the **essentiality of ΔgpsB mutations is suppressed by inactivation of PhpP** protein phosphatase, which concomitantly restores protein phosphorylation levels. In addition, we showed that in pneumococci GpsB forms complexes with PBP2a and PBP2b, and that deletion or depletion of GpsB prevents closure of the septal ring that in itself is PBP2x-dependent **Rued et al (2017) Mol. Microbiol. 103(6), 931-957 (IF = 3.6, Times cited: 20)**. We believe that our contribution was about 30% (four coauthors) and consisted of characterization of suppressor *phpP* mutants

We also participated in the international multi-institutional project that investigated **structural basis of catalysis by the essential PBP2x** of *Streptococcus pneumoniae* by disclosing a total of four X-ray structures, two computational models based on the crystal structures, and molecular dynamics simulations. A prerequisite for catalysis by transpeptidases, including PBP2x, is the **molecular recognition of nascent peptidoglycan strands,** which harbor pentapeptide stems. We disclose that the **recognition of nascent peptidoglycan by PBP2x** takes place by **complexation of one pentapeptide stem at an allosteric site located in the PASTA domains of this enzyme**. This binding predisposes the third pentapeptide stem in the same nascent peptidoglycan strand to penetration into the active site for the turnover events. The complexation of the two pentapeptide stems in the same peptidoglycan strand is a **recognition motif** for the nascent peptidoglycan, **critical for the cell-wall cross-linking reaction**. **Bernardo-García et al (2018) ACS Chem. Biol. 13, 694-702 (IF = 4.4, Times cited: 5**)This work was a result of long-term collaboration with two leading structural biology laboratories; Institute of Physical Chemistry "Rocasolano," at CSIC, Spain and Department of Chemistry and Biochemistry at University of Notre Dame, USA. Our laboratory prepared the

expression systems, optimized purification scheme, and provided crystallization-quality protein. We also s contributed to biological interpretation of the data obtained.

Further, we studied the function of hypothetical protein Spr1057 of S. pneumoniae with unknown function, which we detected as the most highly repressed gene in ΔstkP strain. The functional relationship between *stkP* deletion and repression of *spr1057* is unknown. Sequence comparison revealed that Spr1057 has a significant similarity to YjjG protein of Escherichia coli. The E. coli YjjG protein, a member of the HAD superfamily, exhibits a high-phosphatase activity towards nucleotide monophosphates and functions as a house cleaning phosphatase in vivo. Using in vitro and in vivo approaches we showed that S. pneumoniae Spr1057 is indeed a pyrimidine-specific nucleotidase with high afinity towards noncanonical nucleotides and very likely plays a similar cellular role as does YjjG in E. coli. We demonstrated that Spr1057 forms dimer molecules and present kinetic evidence showing that Spr1057 can hydrolyze 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP) 10-fold more efficiently than 5'-dTMP as a substrate. We found that a mutant lacking the *spr1057* gene exhibited increased levels of 5-BrdU-induced mutations. In contrast to the *spr1057*-deficient strain, wild-type (WT) cells could efficiently obviate the incorporation of mutagenic nucleotides into the DNA and sustain the mutation rate at the invariant level. All these findings suggest that Spr1057, hereafter referred to as PynA (pyrimidine nucleotidase A), plays a similar physiological role in pneumococcal cells as YjjG does in *E. coli* by controlling the levels of mutagenic non-canonical pyrimidine derivatives. (**Ulrych et al (2019) FEBS J. doi: 10.1111/febs.15049 (IF = 4.7, Times cited: 0**)).
In conclusion, we demonstrated by both *in vitro* and *in vivo* approaches that PynA, a novel antimutator protein of *S. pneumoniae*, can efficiently protect the cells from the deleterious effect of mutagenic nucleotide derivatives. Interestingly, in our previous study, the genetic locus encoding the *spr1057* gene and the neighbouring genes was found to be deleted in one class of suppressors of the Δ*gpsB* mutant of the *S. pneumoniae* progenitor strain D39 although no particular function was ascribed to the deletion [**Rued et al (2017)**]. We hypothesize that the relatively high mutation rate associated with deletion of the *spr1057* locus could be responsible for the selection of fast-growing suppressor mutants.
The study was entirely conceived, designed, performed and interpreted by the members of the laboratory research group.

The work of eukaryotic research team supervised by T. Vomastek focusses on the **regulation of the ERK cascade**; in particular how is the ERK signaling, through the phosphorylation of functionally diverse substrates, implemented into specific biological response. The role of the ERK pathway is also investigated in the context of cancer development and cancer progression. In a second project we are investigating **the role of nucleus and nucleus-attached actin fibers on cell polarity, cell migration and invasion**.
We investigate **the role ERK** plays in the establishment of **migratory phenotype** using either fibroblast cells establishing front-rear polarity upon integrin-mediated adhesion or epithelial cells induced to migrate. The research primarily concerned mechanism that control actin reorganization in spreading cells. Cell spreading is a morphogenetic process during which cells break radial symmetry and adopt migratory polarity with well-defined and spatially segregated protruding cell front and non-protruding cell rear. **We showed that the development of protruding and non-**

protruding regions requires scaffold protein RACK1 and kinase ERK2 that both coordinate local depletion of the membrane-associated p190A-RhoGAP and consequently actin stabilization in this region. Since adhesion induces ERK activation and suppression of p190A-RhoGAP membrane localization, our data suggest that cell polarization is controlled by a Turing-like signaling circuit in which p190A-RhoGAP activation is locally suppressed by ERK signaling. These results were published **in Klimova et al. (2016) Biochimica et Biophysica. Acta – Molecular Cell Research 1863, 2189-2200, (IF2016 = 4.5).** This work was entirely done at the Institute of Microbiology. International collaborator provided reagents and contributed to final manuscript editing.

Work performed in parallel concerned the characterization of non-lysosomal protease **Calpain2 as an ERK substrate and the effect this signalling has on epithelial-mesenchymal transition and epithelial cells migration**. Our data suggests that ERK2 phosphorylates Calpain2 and associates with calpain2, however, this interaction is most likely indirect. The ERK and calpain2 signaling affects significantly so called pluricellular actin bundles. These functionally poorly understood contractile actin bundles are assembled at the edge of epithelium. We found that peripheral bundles are between cells coupled to intercellular adhesion puncta enriched in the component of cell-cell junctions including E-cadherin, Vinculin or Tricellulin. The ERK activation or the expression of constitutive active Calpain2 disrupts the peripheral actin and intercellular puncta, and induce mesenchymal-like migration with dominant lamellipodia. Conversely, ERK inhibition or Calpain2 knockout by Crispr/Cas9 system impaired peripheral actin remodelling and promoted purse string mechanism of migration. Our study suggests that **ERK and its downstream effector Calpain2 are sufficient and necessary for the peripheral actin remodelling and regulate the transition between different modes of epithelial cell migration.** The manuscript with these results is under preparation and it will be submitted in 2020.

Obtained results concerning the role of ERK in cell polarity, epithelial-mesenchymal transition and cell invasion allowed us to contribute to the comprehensive review published in Oncotarget (IF2016 = 5.2) (Gandalovicova A., Vomastek T., Rosel D., Brabek J. **Cell polarity signaling in the plasticity of cancer cell invasiveness. Oncotarget 7: 25022-25049 (2016)**). The team's contribution to the manuscript is 25 %.

Our work on ERK signaling resulted in collaboration with group of Prof. L. Vitek from 1st Faculty of Medicine at Charles University. We examined the anti-oxidant and anti-tumor effect of naturally derived compounds, in particular how they affect **pancreatic cancer development and progression**. We contributed to the study examining the anti-oxidant properties of chlorophylls and chlorophyll-like tetrapyrrolic compounds. We found that these compounds display strong anti-oxidant properties without affecting the general signaling such as the activity of ERK and AKT. We have shown that chlorophylls produce antiproliferative effects in pancreatic cancer cell lines and also in mouse in vivo experiments. These findings underline the positive effect of fresh green vegetable diet on human health. These results were published in **Vankova et al. 2018 (Chlorophyll-Mediated Changes in the Redox Status of Pancreatic Cancer Cells Are Associated with Its Anticancer Effects. Oxidative Medicine and Cellular Longevity, Article ID 4069167, (IF2018 = 4.9)**. We contributed by determining the activity of ERK and AKT signaling pathways and

manuscript writing and editing (approx. 10 %). Additional project stemming from the collaboration with Prof. Vitek concerned the anti-tumor effect of alga *Arthrospira platensis*. Using pancreatic tumors as a model, we found that *A. platensis* extract display strong anti-tumor and anti-angiogenic properties both in *in vivo* mouse models and *in vitro* cell lines. The anti-tumor properties can be at least partly attributed to the inhibition of the ERK pathway that was observed upon the treatment with *A. platensis* extract. The extract was effective specifically on the cell lines containing activating mutations in oncogene Ras, but not Raf, indicating that *A. platensis* could have potentially therapeutic effect in pancreatic cancers where Ras mutations are very common. The manuscript containing these results (**Marková et al. (2020) Anti-angiogenic effects of the blue-green alga Arthrospira platensis on pancreatic cancer, Journal of Cellular and Molecular Medicine 24: 2402-2415 (IF2019 = 4.7**) was accepted for publication in 2019, however, printed version appeared later in February 2020. We contributed by determining the effect of extract on activity of ERK and AKT signaling pathways in pancreatic cell lines, designing and performing experiments showing that extract is specific for cells containing Ras mutations and by manuscript writing and editing (approx. 30 %).

We also took part in the multi-institutional project that investigated the pathology of **head and squamous cell carcinoma using tumor samples obtained from patients**. The teams participating in the study include Faculty Hospital in Motol (collection of tumor samples an clinical data), Institute of Anatomy of 1st Faculty of Medicine at Charles University (immunohistochemistry and samples preparation for expression profiles), Institute of Molecular Genetics (microarrays expression profiling, bioinformatics analysis) and Institute of Microbiology (analysis design, biological interpretation of results, experimental confirmation of results). Previously and newly collected samples constitute more than 300 expression profiles of squamous cell carcinoma of the head and neck. The expression profile analyses clearly demonstrated existence of several subgroups of HNSCC with different transcription signatures. Moreover, this dataset contains expression data from 82 tumour samples and paired normal mucosa and represents to our best knowledge the largest collection of paired HNSCC samples accompanied with patient´s clinical data. Using this dataset, we analysed the expression pattern of cyclin D1 as it is a strong oncogene whose overexpression is frequently found in HNSCC. Comparative analysis of normal tissue and paired tumours showed that cyclin D1 was not only upregulated as expected but, counterintuitively, it was in large fraction of tumours often downregulated. Survival analyses showed, however, that there was no correlation between the change in cyclin D1 expression and patient survival. We also showed that a change in cyclin D1 expression is compensated by a change in expression of cyclin D2 which could explain the lack of correlation between cyclin D1 downregulation and better clinical outcome. Manuscript containing the patients dataset, gene expression profiles and Cyclin D1 expression analysis is currently under review in journal **Cancers (IF2019=6.2)** where T. Vomastek is last co-corresponding author (**Novotny et al., Analysis of head and neck squamous cell carcinomas and paired normal mucosae reveals HPV-dependent cyclin D1 deregulation and compensatory effect of cyclin D2**). Although the manuscript contains more than 15 authors, we contributed significantly to the interpretation of the results, design of a new bioinformatics approaches and manuscript writing.

In a second major project in the group, we are investigating **the role of nuclear movement on cell polarity**. Our work focused on functionally poorly understood contractile perinuclear actin fibers, specific sub-type of stress fibers that rise from leading edge above the nucleus and terminate at the cell rear forming dome-like structure. The central part of perinuclear actin fibers is physically attached to the nucleus through **LINC complex.** Perinuclear fibers are organized in parallel bundles that are aligned with the axis of migration and support the adoption of front-rear polarity. Our work revealed the mechanism of perinuclear actin cap formation. Actin cap formation and cell polarization involves dorsal fibers and transverse arcs formed at the cell front and peripheral ventral stress fibers present along the cell sides. These fibers assemble in actin network that is interconnected in foci that are rich in actin and alpha-actinin-1 but devoid of myosin II. This interconnected actin network moves coordinately from the cell periphery toward the nucleus and to the dorsal side of the cell ultimately yielding actin filaments above the nucleus oriented parallel with the front-rear axis of the cell. These results was published in **Maninova et al., FEBS Journal 283: 3676-3693 (2016), (IF 2016= 3.9).** Obtained results concerning the cell polarization and the function of perinuclear actin fibers in nuclear movement resulted in invited review published in journal Protoplasma (**Maninova et al. (2017) Protoplasma. 254:1207-1218 (IF 2016=2.9)**. Both manuscript were entirely prepared in our lab. Our work then focused on the analyzing the mechanism of interaction of perinuclear actin fibers and LINC complex and how they affect the migration and invasion of cancer cells. We have identified the region of LINC complex component nesprin2 that upon expression is able to inhibit cell migration. In this manner, we began to collaborate with J. Hejnar and J. Plachy (Institute of Molecular Genetics in Prague) in order to utilize their avian model of metastatic formation. In this model, the cancer cells are injected into pectoral muscle of syngeneic animals to form primary tumours. To metastasize in distant lung, cancer cells must undergo whole metastatic cascade, i.e. invasion, extravasation, intravasation and metastasis formation. Our data show that disruption of link between nucleus and cytoskeleton completely abrogates the ability of cancer cells to metastasize. We have prepared several expression constructs that should help us to delineate more directly the function of perinuclear actin fibers in invasion and metastatic spread. We plan to publish these results in 2020/2021.

Research activity and characterisation of the main scientific results

We are a basic research team. Hence the value of our research activity should be reflected in the quality of our research papers. The most exciting contributions of our lab to the field in the evaluated period are:

1) In depth characterization of the composition, assembly and function of human translation initiation factor 3 (eIF3).

   Wagner et al., NAR, 2016 (no cooperation); this study outlined for the first time the mechanism of the assembly of human translation initiation factor 3 (eIF3) and illustrated how imbalanced expression of human eIF3 subunits impacts the factor's overall expression profile. As such, it provided a comprehensive guide to the human eIF3 complex and to the relationship between eIF3 misregulation and cancer.

   Herrmannova et. al., NAR, 2019 (no cooperation); this study established a new technique called adapted formaldehyde gradient cross-linking to investigate the efficiency of the assembly of the 43S and 48S preinitiation complexes in knockdowns of individual subunits of human eIF3 known to produce various partial subcomplexes. We revealed what eIF3 subunits contribute to what specific roles in cells and thus provided new insights into the role of human eIF3 in the initial assembly steps of the translational machinery.

   Valasek et al., NAR, 2017 (no cooperation); in this study we reviewed recent structural views of the eIF3–40S complex and discussed all known eIF3 roles to provide a broad picture of the eIF3's impact on translational control.

2) Structural and functional characterization of yeast eIF3 either free in solution or bound by other eIFs or the small ribosomal subunit.

   Zeman et al., NAR, 2019 (cooperation); in this study we showed for the first time that (a) the budding yeast translation initiation factor eIF3 adopts a very compact conformation when free in solution, (b) binding of eIFs 1 and 5 to eIF3 further compacts its globular 3D geometry to expose mainly the ribosomal contact points, (c) eIF3 and eIF5 undergo dramatic structural rearrangements upon their binding to the 40S ribosomal subunit. This study was conceived, designed, carried out, analyzed and written in our laboratory. The group of Petr Novak taught us how to carry out cross-linking, ran the Mass-spec analysis and greatly helped us with Mass-spec data analysis. Yuzuru Itoh optimized the purification protocol.

   Aitken et al., eLife, 2016 (cooperation); this study addressed several key outstanding questions in our understanding of translation initiation, namely how translation initiation factor eIF3 contributes to recruiting the 43S pre-initiation

complexes to the mRNA and stabilizing the resulting complex, and how its individual subunits contribute to these events. This is equal contribution of three co-corresponding labs – hence our share is 30%; generation of many overexpression plasmids and strains, large scale factor isolations, mRNA recruitment analysis in vivo, study design, data analysis, paper writing.

3) Characterization of molecular means enabling the small ribosomal subunit to reinitiate translation on the succeeding ORF occurring on the same mRNA molecule after termination followed by incomplete ribosomal recycling on the preceding ORF under various stressors.

   Mohammad et al., NAR, 2017 (no cooperation); this study revealed for the first time that translation initiation factor 3, which coordinates most of the initiation steps, does not come off the initiation complex upon subunit joining. It remains bound to 80S ribosomes and gradually falls off during the first few elongation cycles. If the translated ORF is short enough to preserve eIF3 bound beyond its termination, eIF3 stabilizes the mRNA-40S post-termination complex to promote its resumption of scanning for reinitiation on downstream ORFs.

   Gunisova et al., RNA, 2016 (no cooperation); this study described in great detail all cis- and trans-acting factors that are required to enable efficient translation reinitiation in the budding yeast.

   Guan et al., Mol Cell, 2017 (cooperation); this study revealed that higher eukaryotic cells respond differently to chronic ER stress versus acute stress on the translational level and that the key role is played by translation initiation factor eIF3, and in particular its eIF3d subunit. Even though we seem to have only a small share on this paper, I think our contribution was significant. We joined later this consortium of various teams and contributed with our know-how and mainly with data interpretation that, according to the last author, greatly helped to get this work finally accepted after several rounds of revisions.

   Gunisova et al., FEMS Microbiol Rev, 2018 (minor cooperation); in this study we reviewed comprehensively all known instances of both short uORF-mediated and long ORF-mediated reinitiation) and presented our current understanding of the underlying molecular mechanisms of these intriguing modes of translational control. The only non-LRGE co-author featuring in the author's list, my former NIH supervisor, contributed intellectually by expanding our horizons of thinking.

4) Identification and functional characterization of the major players contributing to the programmed stop codon readthrough.

Beznoskova et al., NAR, 2015 (minor cooperation); our work uncovered that, unexpectedly, the translation initiation factor 3 (eIF3) plays a critical role in dictating the ribosome to readthrough the stop codon to extend the nascent polypeptide at its C-terminus when programmed. Furthermore, we experimentally verified the longstanding hypothesis that readthrough is enabled by near-cognate tRNAs with a mismatch at the third, wobble position to any of the three stop codons when these are occurring in the termination non-permissive context. The only non-LRGE co-author featuring in the author's list contributed yeast strains, as well as intelectually.

Poncova et al., NAR, 2019 (no cooperation); here we showed that the small ribosomal protein Rps3 controls fidelity of translation termination and programmed stop codon readthrough in co-operation with translation initiation factor eIF3, as the first Rps directly implicated in these processes ever. We also demonstrated that Rps3 modulates the conformation of the mRNA entrance tunnel during translation initiation as well as termination, and promotes incorporation of near-cognate RNAs into the A-site in a stop codon tetranucleotide-specific manner.

Beznoskova et al., NAR, 2019 (minor cooperation); here we identified and described a complete group of yeast tRNAs that induce readthrough in the stop-codon tetranucleotide manner when overexpressed; designated readthrough-inducing tRNAs (rti-tRNAs). We also developed Yeast Applied Readthrough Inducing System (YARIS), a reporter-based assay allowing to simultaneously detect readthrough levels at all twelve stop-codon tetranucleotides. Finally, we employed YARIS to study the effects of natural tRNA modifications and readthrough-inducing drugs on readthrough. The only non-LRGE co-author featuring in the author's list contributed intellectually (advice on data presentation).

Beznoskova et al., RNA, 2016 (no cooperation); this study revealed the mechanism and rules of how some near-cognate tRNAs increase the efficiency of stop codon readthrough in the stop codon-specific manner in the budding yeast.

Research activity and characterisation of the main scientific results

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

We found that the key PSII protein D1 is synthesised, matured and attached to the partner D2 in thylakoid membrane (TM). These results disprove an earlier hypothesis that these processes take place in the cytoplasmic membrane (PM). However, we propose that an interaction between the TM and PM at specific points, either permanent or temporary, is necessary for efficient TM biogenesis and PSII assembly. We provided the key CP47-less strain and member of the laboratory J. Knoppová participated in the purification of plasma and thylakoid membranes from this strain and wild-type in the Singapore laboratory which provided the methodology for purification. The purified membranes were transported to our laboratory and they were analysed using 1D and 2D gel electrophoresis and western blotting. We also significantly contributed to writing of the manuscript. (Selão et al., Plant and Cell Physiology 57, 95–104, 2016).

We elucidated the function of a distant cyanobacterial homologue of the eukaryotic PsbP protein, termed CyanoP. While the PsbP is one of the constitutive extrinsic subunits stabilizing the oxygen-evolving Mn_4CaO_5 cluster of photosystem II (PSII) in plants, the CyanoP plays a role of an assembly factor involved in the early steps of PSII biogenesis. The vast majority of work has been done in laboratory 131 including construction and characterization of strains, pigment and protein analyses, radioactive labelling, isolation and purification of the protein. Partners from Imperial College provided the CyanoP-less strain and participated in the last stage of writing (Knoppová et al., Plant and Cell Physiology 57, 1–11, 2016.

We demonstrated that the auxiliary factors Psb27 and Psb28, which we previously found to be bound within PSII become components of supercomplexes formed by the non-oxygen evolving photosystem II and photosystem I (PSII-PSI) when the cells of the cyanobacterium Synechocystis 6803 are exposed to increased irradiance. Formation of these associations may be an important step in PSII biogenesis especially under photoinhibitory conditions possibly photoprotecting PSII through energy spillover. The study was entirely planned and performed in the laboratory, collaborators from Imperial College just provided the set of psb27, psb28-1 and psb28-2 null mutants while electron microscopy was performed at the Faculty of Science, University of South Bohemia (Bečková et al.. Molecular Plant 10, 62-72, 2017).

We identified the cyanobacterial Pam68 protein as a ribosomal factor that is in contact with the nascent CP47 subunit of photosystem II in the vicinity of the SecY protein translocase. Based on our results we suggest that Pam68 facilitates the insertion of chlorophyll molecules into the translated CP47 polypeptide chain. Furthermore, our findings support the existence of a pool of modified, stress-induced type of ribosomes in cyanobacteria. The work was entirely done in the laboratory (Bučinská et al. Plant Physiol. 176, 2931–2942, 2018).

We characterized in detail function of PSII assembly factor Ycf48 and obtained its high resolution structure. We proposed a model for the function of Ycf48 in binding of chlorophyll to apoprotein during insertion of chlorophyll-binding proteins into the membrane. We showed that Ycf48 is a seven-bladed beta-propeller with a highly conserved functionally important arginine patch. The biochemical characterization of various Ycf48 strains, electrophoretic analyses, labeling, isolation of Synechocystis

proteins and complexes and their analyses were done in the laboratory; determination of the Ycf48 structure and construction of site directed mutants was done by collaborators from Imperial College (Yu et al. PNAS 115, E7824–E7833, 2018).

We found that sequential deletions of Ycf48, Ycf39 and Pam68 lead to the gradual loss of autotrophy accompanied by loss of OPSII and chlorophyll. The entire work was done in our laboratory (Knoppová and Komenda, Folia Microbiologica 64, 683-689, 2019).

We discovered that rubredoxin A (RubA), a protein vital for photosynthesis with a likely role in transporting electrons within the cell, plays a role at an early stage in photosystem II (PSII) biogenesis, during formation of the D1-D2 reaction centre complex.. We suggest that it may keep PSII cofactors in a proper redox state during assembly to prevent photodamage and disassembly. We also demonstrated an indirect effect of the lack of RubA on the accumulation of photosystem I. The majority of work was done in the laboratory including construction and characterization of strains, pigment and protein analyses, radioactive labelling, isolation and purification of the protein. Partners from Imperial College provided the rubredoxin-Ycf48 fusion strain and participated in the last stage of writing, Petr Halada did mass spectrometric analysis of the FLAG-RubA preparation (Kiss et al. Plant Cell 31: 2241–2258, 2019).

We found that the cyanobacterium *Synechocystis* can significantly improve the efficiency of PSII assembly and consequently viability by introducing tandem duplications of large chromosomal regions. Work was completely done in our laboratory (Tichý et al. Frontiers in Plant Science 7, 648, 2016).

We constructed the Synechocystis strain lacking the large PSI protein PsaA and expressing FLAG-tagged PsaB. Besides a number of PI assembly factors, which we co-purified with it, the preparation also contained D1 and D2 proteins and PSII assembly factors. These results support our hypothesis about the modular character of PSI assembly and the intertwined biogenesis of both photosystems.

We were characterizing the early D1-D2 RCII assembly complexes. We identified new protein components associated with these complexes and also found unique complex of RCII with a unusual PSI monomer with low chlorophyll content. The complexes showed heterogeneity in both pigment and protein composition and reasons for it require further study.

We identified two specific complexes of PSII with chlorophyll biosynthesis enzymes unassembled CP47 with chlorophyll cyclase and RC47 assembly complex with cyclase and protochlorophyllide oxidoreductase. These results justify the observed depletion of chlorophyll in cyanobacterial mutants lacking CP47.

We characterized mutants with the PSI-like PSII complex in which the psbA is merged with psbC and psbB with psbD to attach internal PSII antennas CP43 and CP47 to the reaction centre proteins D1 and D2, respectively. Under higher irradiance the mutants suffered from strong photoinhibition and reverse secondary mutations frequently appeared which decreased the light sensitivity of the strain. Some of them were identified and appearance of the extra copies of the separate D1 protein was proved in the genome supporting our hypothesis that the reason for the sensitivity is the impairment/retardation of the PSII repair. Other revertants are being sequenced.

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

We showed that the Psb29 protein originally considered a PSII assembly factor of the unknown function is, like *thylakoid formation1* factor (THF1) in *Arabidopsis*, important for normal accumulation of the FtsH2/FtsH3 complex involved in photosystem II (PSII) repair, and that it physically interacts with FtsH complexes *in vivo*. So, its effect on the PSII is only indirect via effect on the PSII repair and Psb29 should not be considered PSII assembly factor. To gain further insights into Psb29, we have determined the crystal structure of Psb29 encoded by *Thermosynechococcus elongatus*, a thermophilic cyanobacterium widely used to study structural aspects of PSII assembly and repair. We performed characterization of the psb29-null strain, its transcript analysis, isolation of flagged Psb29 and FtsH2 and their analysis by 2D SDS gel and blotting, so our work was crucial for establishing the function of the protein. Collaborators from Imperial College constructed the Psb29 null and flagged strains and determined the structure of the protein (Bečková et al. Phil. Trans. R. Soc. B. 372, 20160394, 2017).

We demonstrated that recognition and subsequent degradation of key PSII proteins D1 and D2 occurs once these reaction centre proteins become accessible to the specific FtsH2/FtsH3 protease complex, even in the dark when no photooxidative damage occurs. Nevertheless, the light-induced photoinactivation of PSII can cause destabilization of binding/ detachment of the antenna CP43 shielding D1 which thereby directs the protease towards its degradation. The entire work was done in Třeboň, collaborators from Imperial College provided control FtsH-less mutants and participated in writing (Krynická et al. Nature Plants 1, 15168, 2015).

We showed that the essential heterocomplex of membrane FtsH proteases FtsH1/FtsH3 located in cytoplasm plays a much more extensive role in the regulation of cyanobacterial response to nutrient stress (iron, nitrogen, carbon or phosphorus depletion) than previously thought. We demonstrated that the FtsH1/3 functions upstream of individual transcription factors controlling their level and in this way also the activity of stress-induced regulons and thus enable fast tuning of the cellular responses to environmental stimuli. Majority of work was done in our laboratory including experimental design, construction and characterization of FtsH knock-down strains, protein gel analyses, evaluation of transcriptomic and proteomic data and most of writing. Partners from Sheffield University collected and partly evaluated the proteomic data, from University of Freiburg and Algarve collected the transcriptomic data. (Krynická et al. Plant Cell 31, 2912–2928, 2019).

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

We found that a complex impact of phosphatidylglycerol (PG), an only lipid completely essential for the oxygenic photosynthesis, on chlorophyll (Chl) metabolism. The diminished rate of Chl formation was accompanied by impaired synthesis of photosystem I (PSI) complexes. Moreover, the PG-depleted cells were not able to reutilize Chl. We discuss a scenario that the Chl biosynthesis and synthesis of core PSI subunits are colocated in PG-enriched membrane microdomains. The vast majority of work has been done in our laboratory including design of experiments, characterization of strains, pigment and protein analyses, precursor analyses and radioactive labelling. Partners from BRC provided the PG-less strain and PG analysis of the strains was performed at the Biology Centre in České Budějovice (Kopečná et al. Plant Physiology 169, 1307-1317, 2015).

We identified an important structural/functional features of Gun4, a porphyrin-binding protein known to stimulate in vitro the magnesium chelatase activity. We

used in silico docking of protoporphyrine and based on results we characterized mutants with site-specific mutations in the protein which affected its interaction with magnesium chelatase. The biochemical analyses of the mutants were performed in our laboratory (Kopečná et al. Journal of Biological Chemistry 290 (47), 28477-88, 2015).

We successfully deleted the ycf54 gene encoding a component of the oxidative cyclase involved in chlorophyll (Chl) synthesis, which was only possible in a specific substrain of the cyanobacterium Synechocystis. We provide clear evidence that the Ycf54 protein is important, but not essential, for activity of the cyclase. The dramatically reduced Chl formation in this mutant allowed us to demonstrate that the requirement for de novo Chl molecules differs among individual Chl-binding protein. We initiated the work by the complete segregation of the Ycf54 mutant, we performed spectroscopic, electron-microscopic and 1D/2D electrophoretic analysis of the mutant including Western blots and radioactive labeling. Our partners in Sheffield contributed by providing unsegregated strain and MS-and NMR analyses of pigments (Hollingshead et al. Frontiers in Plant Science 7, 292, 2016).

We discovered that the yet uncharacterized cyanobacterial isoform HemJ of the protoporphyrinogen oxidase (PPO), the last enzyme that is common to both chlorophyll and heme biosynthesis pathways, is a new b-type heme protein functionally coupled with coproporphyrinogen III oxidase. The isolation and almost complete characterization of the protein was done in our laboratory 131, only mass spectrometric identification of harderoporphyrinogen was performed in the laboratory 132, while Mark Shepherd from University of Kent participated in the interpretation of spectroscopic data (Skotnicová et al. Journal of Biological Chemistry 293(32), 12394-12404, 2018).

We provided evidence that the conserved transmembrane segment of the cyanobacterial ferrochelatase (FeCh) binds chlorophyll and carotenoids organized in an energy dissipative conformation. We further showed that the FeCh can exist *in vivo* as a pigment-free monomer or a pigment-binding dimer depending on its activity. Our phylogenetic analysis suggested that the protein originated by a fusion between FeCh and a single-helix, high light-inducible protein early in the evolution of cyanobacteria. The work was entirely done in our laboratory, only the evolutionary tree was constructed by Jan Mareš from the Biological Centre in České Budějovice (Pazderník et al. Journal of Biological Chemistry 294(29), 11131-11143, 2019.).

In the research of efficiency of photosynthetic apparatus and its regulation in various microorganisms we obtained the following results:

We experimentally proved the presence of blue-light induced state transitions (STs) in cryptophyte alga, Guillardia theta; which is a well-known mechanism regulation of light-redistribution between Photosystem I and II. Thus, G. theta was found to be the first type of chromalveolate alga (algae from red-clade of photosynthesis) with active STs. Furthermore, molecular mechanism of STs has been proposed based on the reversible nanometer-scale coupling/uncoupling of antennae to/from photosystems. The majority of work has been done in our laboratory: design of experiments, data measurement and analysis, interpretation and most of manuscript writing. All experiments were done in the laboratory by post-doc (O.C.) from partner Umeå University (Cheregi et al. Journal of Experimental Botany 66 (20), 6461-6470, 2015).

We performed the first comparative spectroscopic investigation of Photosystem I complexes isolated from red clade algae. Excitation energy transfer

was measured in PSI from Chromera velia, an alga possessing a split PsaA protein, and from diatom Phaeodactylum tricornutum. The estimated effective photochemical trapping time was in the 15–25 ps range, i.e. twice as fast as in higher plants. The algae analyzed here carried the most efficient charge separation so far reported for isolated Photosystems. We initiated the study, designed the experiment, grew cells and isolated PS1 particles. We have participated in measurements in Italy, in data analysis and in manuscript writing (Belgio et al. BBA-Bioenergetics 1858(1), 56-63, 2017).

We performed in silico and biochemical studies of Photosystem I from the chromerids Chromera velia and Vitrella brassicaformis, autotrophic relatives of apicomplexans. The biochemical data obtained in our laboratory using 2D gel electrophoresis showed unique structural changes especially within PSI which lost several canonical subunits, while PSI gained one superoxide dismutase (Vitrella) or two superoxide dismutases and several unknown proteins (Chromera) as new regular subunits (Sobotka et al. Scientific Reports 7, 13214, 2017).

We developed an improved method for the isolation of pure chlorophyll a/c antenna proteins from the model cryptophytic alga Rhodomonas salina. Antennas were used for in vitro quenching experiments in the absence of xanthophylls, showing that protein aggregation is a plausible mechanism behind non-photochemical quenching in R. salina. Using this method we also purified a functional photosystem I supercomplex, which was characterized by steady-state and time-resolved fluorescence. These methods showed a remarkably fast photochemical trapping rate, similar to what recently reported for other red clade algae such as Chromera velia and Phaeodactylum tricornutum (Kuthanová Trsková et al. Physiologia Plantarum 166(1), 309-319, 2019).

We have solved the mechanisms of diel dynamics of Photosystems I and II (PSII) in cyanobacterium Crocosphaera watsonii, a unicellular diazotroph of global ecological importance that provides nitrogen to aquatic foodwebs in oligotrophic regions of ocean. We show that the nocturnal decline in PSII activity results from monomerization and disassembly of majority of PSII, which is accompanied by incorporation of the non-functional version of the D1 protein, rD1, into small PSII fraction.(Masuda et al. Env. Microbiol. 20, 546-560, 2018).

In the research of mechanisms of non-photochemical quenching we obtained the following results:

We obtained data supporting to the yet unclear origin of the red fluorescence state of CP47 antenna of photosystem II (PSII). The results confirmed an essential role of PsbH subunit of PSII in hydrogen-bonding of the specific peripheral chlorophyll ligated by CP47 proposed here to be responsible for the red fluorescence state possibly playing role in non-photochemical quenching. Construction of the cyanobacterial strains, isolation and spectroscopic measurements of the CP47 antennae, electrophoretic analyses, pigment analyses and partially writing and figure preparation were performed in our laboratory. Partners from University Amsterdam isolated PSII complexes containing and lacking PsbH, measured their 77K spectra and participated in writing (D'Haene et al. BBA-Bioenergetics 1847, 1327-1334, 2015).

Employing a newly characterized sub-complex of the cyanobacterial ancestors of plant antennae HliD/HliC we provided the proof of principle that the carotenoid-induced Chl-*a* light energy quenching achieved via direct energy transfer can operate in the light-harvesting complexes (LHCs) in plants. The mechanism of LHC-based

dissipation may be an ancient cyanobacterial invention redesigned by algae and plants for the control of light harvesting and prevention of the light-induced damage of photosynthetic apparatus. We initiated the study and provided the unique preparation of the Hlip complex for the spectroscopic measurement in the collaborating laboratory at Faculty of Science of University of South Bohemia. We also tested a possible pH-depending quenching in the preparation. (Staleva et al. Nature Chem. Biol. 11, 287-292, 2015).

In the following study we extended the understanding of the role of cyanobacterial high-light-inducible proteins (Hlips) in protection against the light-induced damage. Ultrafast transient absorption spectroscopy applied to the isolated chlorophyll-synthase (ChlG) - Hlips complex revealed efficient quenching of Chl-*a* via the energy transfer mechanism. The Chl-*a* quencher was identified as β-carotene bound to Hlips part of the complex. We constructed the strain, we performed the isolation of the unique preparation of the chlorophyll synthase, its purification by gel filtration and characterization by pigment and protein analyses, and we participated in the writing. The preparation was subsequently characterized by femtosecond spectroscopy in the partner St. Louis laboratory and these data were also partly interpreted at the Faculty of Science in České Budějovice (Niedzwiedzki et al. BBA-Bioenergetics 1857, 1430-1439, 2016).

We provided the isolated HliD complexes also for the study of as β-carotene twisting which was performed in the in collaborating laboratory in Université Paris-Saclay (Llansola-Portoles et al. Journal of Biological Chemistry 292 (4), 1396-1403, 2017).

We also isolated and purified another member of Hlip family, HliC, which takes part in assembly of PSII, particularly under stress conditions. This was done in our laboratory, our collaborators from Université Paris-Saclay characterized HliC using low temperature absorption and Raman resonance spectra. Based on the characterization, we made a model of the complex which binds 2 molecules of as β-carotene and four of chlorophyll based on (Shukla et al. Photosynthesis Research 137(1), 29-39, 2018).

We also analysed HliC employing the watermarked femtosecond stimulated Raman spectroscopy to follow the time evolution of the vibrational modes. We concluded that this method constitutes a promising experimental approach to assess energy transfer and quenching mechanisms in oxygenic photosynthesis. We again provided the isolated and purified small HliC protein for its characterization using watermarked femtosecond stimulated Raman spectroscopy performed in the collaborating laboratory at Vrije University in Amsterdam. Tomáš Polívka from Faculty of Science of University of South Bohemia participated in the interpretation of the data. (Hontani et al. J. Phys. Chem. Lett. 9(7), 1788-1792, 2018).

We described different strategies of photoprotection in different organisms (plants, algae). Our data (*in vivo, in vitro, in silico*) identified the sensitivity of antenna proteins to protons as a key factor in the light-harvesting efficiency. The more/less sensitive proteins are to protonation, more/less they are “ready for light induced photo-protection”. Plants antennae are thus better suited for light-harvesting and required additional protein factors (e.g. PsbS) for effective photoprotection. The work was fully done in our laboratory, our long-term collaborator A.V. Ruban from QMU London was involved in the interpretation of data and discussion (Kuthanová Trsková et al. Journal of Experimental Botany, 69 (18), 4483–4493, 2018).

We characterized physiological response the microalga Chromera velia grown to high ligh includes a reduction of PSII number, partial uncoupling of antennas from

PSII and enhanced NPQ while photoinhibition is minimized. The work was fully done in our laboratory (Belgio et al. Photosynthesis Research 135(1-3), 263-274, 2018).

We also evaluated photoprotective strategies in the motile cryptophyte alga Rhodomonas salina. We concluded that besides the protective role of NPQ, the motile R. salina also minimizes high light exposure by increased cell velocity. The work was fully done in our laboratory (Kaňa et al. Folia Microbiologica 64(5), 691-703, 2019).

In the research of dynamics and mobility of photosynthetic proteins and their complexes we obtained the following results:

We described heterogeneity in the PSI, PSII and phycobilisomes distribution in cyanobacteria thylakoids into microdomains by applying a new image processing method suitable for the Synechocystis PCC 6803 strain with yellow fluorescent protein-tagged PSI. this was exclusively performed in our laboratory (Konert et al. Physiologia Plantarum 166(1), 264-277, 2019).

Using this method we newly described organization of thylakoid membrane in cyanobacteria on single cell level. Our model is based on confocal data and depicts thylakoid membrane as a stable heterogeneous mosaic formed by microdomains (Microdomains = heterogeneous membrane areas defined by typical ratios of pigment-proteins). These areas are very stable (in minutes), small (~0.5–1.5μm) and might represent evolutional and functional precursor of the granal/stromal heterogeneity in plant thylakoids. The entire work was done in our laboratory (Strašková et al. BBA-Bioenergetics 1860, 148053, 2019).

In relationship to applied research and optimalization of instrumentation used for the photosynthesis research we obtained the following results:

We optimized the use of Nannochloropsis for further biotechnological studies. Work was done in our laboratory, collaborators performed cell sorting and gave some recommendations during experiments (Noda et al. Journal of Applied Phycology 29(2), 853-863, 2017).

We optimized the fluorescence labelling method of *Chlamydomonas reinhardtii* cells using quantum nanodots based on silicon compounds (CdSe/ZnS) (Elzorkany et al. Science of the Total Environment, 666, 480-489, 2019).

We optimized the confocal microscope Zeiss available in our laboratory for studies of photosynthetic microorganisms (Steinbach and Kaňa, Microscopy and Microanalysis 22, 258–263, 2016).

We developed automated software for evaluation of 2D electrophoretic gels.

We adapted methodology of sample fixation for automatic photoactivation (based on Arduino processor) during confocal microscopy (hardware/software synchronization with the microscope Zeiss-LSM880/ZEN Black.

We developed programmable LED source of light for the orbital shaker used for cyanobacterial cultivation.

We developed a new cultivation apparatus with direct Peltier cooling.

We developed an apparatus for intensive radioactive labelling of cyanobacterial and algal cells.

Research activity and characterisation of the main scientific results

In most of the research direction the substantial progress has been achieved during the evaluation period, although in some cases this was not always reflected in the publication activity of the laboratory due to the two reasons. (1) some of the initiated studies were planned as long term with a strong output (publication/patent) after finalizing all the data (designated below as: ongoing project), (2) the research team has substantially changed its structure as many young researcher has been acquired and the head of the laboratory has been changed in 2016.

In the field of the bioactive secondary metabolites we have finalized the middle size screening of the cyanobacterial crude extracts (-150 in total) for cytotoxic and proapoptotic activity. We have identified several compound with interesting bioactivity either from toxicological (lipopeptides - muscotoxins and puwainaphycins) or potentially pharmacological point of view (nocuolin A, 7-OH tolytoxin and metabolite 2504). Concerning our long term goal, we continued with addressing the biological activity of cyanobacterial lipopeptides and found that both studied classes (muscotoxins and puwainaphycins) are targeting mainly eukaryotic cell membrane and that they cause increased membrane fluidity (puwainaphycins) or membrane stiffening (muscotoxins) followed by calcium leakage into the cell eventually leading to the necrotic cell death (Tomek et al. 2015). Further we have found that some of the isolated lipopeptide variants have promising antifungal activity against important plant pathogens and thus we have developed methodological platform for their isolation. In the part devoted to compounds with potential anticancer activity we have fully elucidated the structure of the first natural oxadiazine nocuolin A and find out that it is causing the caspase-dependent apoptosis in human cancer cells. The initial screening also led to isolation of novel metabolite 2504 causing cell cycle arrest in human cancer cells at nanomolar ranges and thus it is extremely interesting for further studies. The compound was tested on the panel od 25 cancer lines and 3 human primary cell lines and we have noted interesting selectivity against particular cancer models (especially breast cancer) which further enhanced our interest in the compound (ongoing project). The compound mechanism of the action was assessed using metabolomics and proteomic approaches and genetic Crisp/Cas9 screens (ongoing project). In the third compound with anticancer potential (7-OH tolytoxin) we have isolate the compound in the pure state and we have studied the cell death modality in different cell lines treated with the compound. The results indicated that compounds of tolytoxin family are causing caspase-dependent apoptosis and might be interesting for studies aiming the targeting of these molecules to the cancer cells. At the end of the 2019 we have finished the preparation of the fraction library of 1600 fractions of crude cyanobacterial extracts for future high-throughput screenings and initiated the screening.

In the field of cyanobacterial secondary metabolite biosynthesis, we have characterized the biosynthetic gene clusters from various lipopeptide producers and have found the mechanisms by which the fatty acid moiety variability within the structure is achieved (Mareš et al. 2019). These findings are extremely important especially taking into account that the fatty acid moiety is playing key role in the compound bioactivity and thus these results are setting a base for future modification of the compound. We have also developed a bioinformatics pipeline to screen for

lipopeptide biosynthetic gene clusters in cyanobacterial genomes and we found occurrence of such clusters in 16% of cyanobacterial genomes, suggesting their frequent occurrence and predominance in biofilm-associated strains. The genomic screening has also revealed new type of lipopeptides possessing hydroxyl-aspartate moiety which we successfully isolated and provide experiential evidence on their physiological role as siderophores (ongoing project). Finally, we co-operated in clarifying of the biosynthetic gene clusters and biosynthesis routes in three bacterial metabolites – namely cusperin (Kust et al. 2019), kawaguchipeptin and neoenterocin. All these studied were performed in collaboration with other institutions, however, in all of them our expertises in compounds isolation and bioactivity assessment played an important role. Most importantly we have elucidated the structure of pederin analog cusperin isolated from planktic cyanobacterium *Cupidothrix issaschenkoi* which is of special interest as pederin family compounds are potent ribosomal poisons, until recently thought to be exclusively produced by bacteria in symbiotic association with eukaryotes (insect guts, lichens). Our study demonstrates the production of a novel pederin-family compound by a widespread, free-living, and bloom-forming cyanobacterial species. Moreover, we have found that cusperin is lacking typical cytostatic activity typical for other pederin-class compounds, which is interesting for the structure-activity relation studies.

Within the activities connected to the physiology of algae in phototrophic cultures the physiological characterisation and subsequent optimization of the growth conditions has been performed for several biotechnologically important strains (*Chlorella fusca*, *Nostoc*, *Cylindrospermum allatosporum*, *Pseudosspondiococcum*) and we have proposed optimal conditions for their large scale cultivation including the biphasic approaches to enhance the production of compounds of interest (Jerez et al. 2016). The working group of phototrophic cultivation further invested big effort in the experiments using the waste water to grow algal strain with phyto-stimulating and antifungal potential (ongoing project) as this is the main goal of the international consortium SABANA (ongoing project, see below). In a joint effort with heterotrophic group the experiments leading to study the accumulation of seleno-aminoacids under photo- and hetero-trophic regimes has been performed. The obtained biomass was studied for the bioaccessibility of the selenium for humans. The study led to important finding that disintegrated Chlorella biomass contains high content of selenoaminoacids which are highly bioaccessible (Vu et al. 2019).

The sub-group of biorafinery and downstream processing has been intensively working on the optimization of the extraction platform for isolation of valuable algal carotenoids (lutein, astaxantin and fukoxanthin) and cyanobacterial lipopeptides (muscotoxins and puwainphycins). As the highlight of these activities the purification platform for lutein and astaxathin using the a multiinjection mode was developed which is making the methodological platform scalable and interesting for industrial use. The methodological platform for isolation of astaxanthin esters was developed in a tight cooperation with the Czech company Aveflor as the main goal of the TAČR project and is planned to be used in practise (the corresponding patent is currently under preparation, ongoing project).

The heterotrophic group activities were mostly connected to fulfilling the goals of the large institutional projects National Program of Sustainability (NPU II), National Centres of Competence and MPU-TRIO. Within these projects a development of

cultivation protocols for heterotrophic cultivation of a green alga Chlorella, its mutant with high content of lutein and DHA-rich *Schizochytrium* strains were the main goals. These goals were achieved and requested final products were delivered to the project partners. Aside from the project-related activities the group has optimized the down-stream processing protocols for spray-drying of the *Haematocuccus* and *Nanochloropsis* biomass as a subcontract for the companies BDI and Ecoduna (both Austria).

Research activity and characterisation of the main scientific results

Below are outlined the multiple fields that have been covered by research of the laboratory in the past years.

Coordination between growth and cell cycle

The research topic has been studied in the framework of a Czech Science Foundation projects no. 15-09231S “Growth and cell cycle - mechanisms of mutual coordination” and 19-12607S “Growth and cell cycle - regulation of cell cycle entry and exit” in collaboration with Dr. J. Dresler and Dr. P. Pajer of Military Health Institute and Dr. J. Fulnečková of Institute of Biophysics of the Czech Academy of Sciences.

The cell cycle and growth are intricately intertwined and appear to be coordinated so that entry into cell cycle, which will inevitably lead to cell division, is only possible if critical cell mass, manifested as critical cell size, is reached. In green algae dividing by multiple fission, reaching critical cell size will lead to the attainment of a commitment point (CP), equivalent of Start in yeast. This will lead to completion of one round of DNA replication, and nuclear and cellular divisions. As the mechanism responsible for the coordination between growth and cell cycle entry remains enigmatic, the main aims were to: 1) identify the metabolic signal/s required for cell cycle progression, 2) identify how the cell cycle machinery perceives and translates the signal in order to progress through the cell cycle. To identify the mechanism coordinating cell growth and cell cycle progression we made use of two distantly related green algae species, *Desmodesmus* (formerly *Scenedesmus*) *quadricauda* and *Chlamydomonas reinhardtii*, dividing by multiple fission. Different algal lines were analyzed under several sets of conditions, particularly conditions varying in cultivation temperature as it has been known that critical cell size is affected by temperature. Furthermore, we studied the transcriptome and proteome of cells just attaining CP and searched for genes/proteins that are specifically upregulated and might thus be functionally connected to CP attainment.

1. **Coordination of growth and cell cycle at shifts between physiological temperatures**

Progression of the cell cycle in green algae dividing by multiple fission is, under otherwise unlimited conditions, affected by growth rate set by a combination of light intensity and temperature. We compared cell cycle characteristics of *Desmodesmus* (formerly *Scenedesmus*) *quadricauda* at 20 °C or 30 °C and shifts between them. The duration of the cell cycle in cells grown under continuous illumination was more than doubled at 20 °C compared to 30 °C, suggesting it was set directly by growth rate without temperature compensation of cell cycle was involved. Similarly, DNA, RNA and bulk protein content per cell at 20 °C were about double that of cells grown at the higher temperature. For shift experiments, cells grown at either temperature were transferred to darkness to prevent further growth, and cultivated at the same or the other temperature. Upon transfer to the lower temperature, fewer nuclei and daughter cells were produced and not all cells were able to finish the cell cycle by division, remaining multinuclear. Correspondingly, cells put into the dark at the higher temperature divided faster into more daughter cells than control cells. These differences correlated with shifts in the preceding CDK activity, suggesting that cell cycle progression was not related to growth rate or cell biomass but correlates with CDK activity (Zachleder *et al*., 2019b). This, rather surprisingly, suggests there is no direct interconnection between growth and cell cycle progression and the two processes merely correlate under stable conditions.

2. Cell cycle progression block at high temperature

Increase in temperature within a certain range will increase growth rate and consequently shorten the cell cycle. However, further increase above certain threshold will have detrimental effect on cell. High temperature (minimum of 39 °C) inhibited the cell cycle in *Chlamydomonas reinhardtii,* and in synchronized cultures, nuclear and cellular divisions were blocked completely while DNA replication was partly affected. In contrast, growth (cell volume, dry matter, total protein and RNA) remained unaffected and starch accumulated to very high levels (up to 90% of dry mass). Thus, growth is less sensitive to inhibiting effect of high temperature than is cell cycle progression. Cell cycle arrest was accompanied by high mitotic cyclin-dependent kinase activity that decreased after completion of nuclear and cellular division following transfer to the permissive temperature. Cell cycle arrest was therefore not caused by a lack of cyclin-dependent kinase activity but rather a blockage in downstream processes (Zachleder *et al*., 2019a). Furthermore, the data suggest existence of another, yet unknown mechanism (an inhibitor, temperature labile activator of CDK), directly connecting an increase in nuclear and cellular division with a temperature dependent increase in CDK activity as it was suggested by our experiments with *Desmodesmus quadricauda*.

3. Effect of DNA damage on the cell cycle progression

Once the cells enter the cell cycle, the progression could be affected by various stress factors apart from temperature. DNA damage is a ubiquitous threat endangering DNA integrity in all living organisms. Responses to DNA damage include, among others, induction of DNA repair and blocking of cell cycle progression in order to prevent transmission of damaged DNA to daughter cells. DNA damage caused by UV irradiation is known to decrease cyclin-dependent kinase activity, which leads to a cell cycle arrest. We tested the effect of the antibiotic zeocin, inducing double stranded DNA breaks, on the cell cycle of synchronized cultures of the green alga *Chlamydomonas reinhardtii*. After zeocin application, DNA replication partially occurred but nuclear and cellular divisions were completely blocked. This way the application of zeocin in several aspects mimicked the effect of high temperature. Application of zeocin combined with caffeine, known to alleviate DNA checkpoints, decreased cell viability significantly. This was probably caused by a partial overcoming of the cell cycle progression block in such cells, leading to aberrant cell divisions. The cell cycle block was accompanied by high steady state levels of mitotic cyclin-dependent kinase activity. The data indicate that DNA damage response in *C. reinhardtii* is connected to the cell cycle block, accompanied by increased and stabilized mitotic cyclin-dependent kinase activity (Čížková *et al.*, 2019). This is very similar to the behavior of mitotic kinase activity at 39 °C and it may imply that the effect of high temperature could be partly related to high temperature causing DNA damage or affecting DNA repair.

4. Transcriptome and proteome of CP

Transcriptome of synchronized *C. reinhardtii* cultures in diurnal cycle has been analyzed but the data set contains both light inducible genes and genes specific to attainment of CP and their separation is not possible. To analyze transcriptome and proteome specifically related to attainment of CP we used two different growth conditions. One at standard high light conditions (500 μmol m^{-2} s^{-1}), the other at lower light irradiance (100 μmol m^{-2} s^{-1}). In high light conditions, the CP was attained two

hours after the cultures were illuminated. In the lower light conditions, the attainment of CP was delayed until 9 hours after illumination. In both conditions, we sampled four biological replicates right before attainment of CP (pre-CP), at mid-point of CP attainment (CP) and immediately after CP was attained (post-CP). Transcriptomes from different time-points were analyzed by Illumina sequencing. Proteomes from the same time-points were analyzed by label-free quantification of proteins in collaboration with colleagues from the Military Health Institute. In the set of genes/proteins significantly differing among the conditions, there were many genes and proteins differently expressed between pre-CP and CP (as well as post-CP) samples in high light conditions. In contrast, there is only a small proportion of genes/proteins differentially expressed between pre-CP and CP (as well as post-CP) samples in low light conditions. This way, we were able to identify light induced genes and proteins. This data set included many metabolic genes as well as histones and some ribosomal proteins. The comparison of genes specifically differentially expressed at CP in both growth conditions identified some metabolic genes, chlorophyll binding proteins, plastid and nuclear ribosomal proteins, ATPases, phosphatases, receptors, etc. The most stringently re-testing proteins include subunit of magnesium chelatase, plastid ribosomal protein L9, mitochondrial ATPase associated protein, a component of chloroplast translocation machinery for light harvesting protein and a subunit of phytoene desaturase. The protein selection suggests a correlation of the general and organellar metabolism to CP attainment. We have raised algal lines expressing inducible amiRNAs (artificial micro RNA) targeted against these genes and we have been analyzing their phenotypes.

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Algae grown in deuterated water

The research topic was studied in the framework of a Czech Science Foundation project no. 17-06264S “Growth and division in stable isotopes – beyond metabolic labeling” in collaboration with Dr. P. Mojzeš from Institute of Physics, Charles University. The aim of the project was to identify how algal cells dividing by multiple fission incorporate deuterium, and to characterize the mechanisms allowing them to grow and divide even at very high concentrations of heavy water.

We have found that cultivation in heavy water causes a concentration dependent slow-down in growth. At 70 % D_2O, the growth of three green algae, *Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Desmodesmus quadricauda* is slowed down to about half. At 90 % D_2O, the growth is again slowed to about half. Cultivation in heavy water affects numerous metabolic processes thus causing stress. Lowering of the light intensity decreases the growth rates and allows the cells to survive higher concentration of D_2O. In line with this, the mutants of *C. reinhardtii* with improved “growth in D_2O” (GDO) show a general metabolic slow-down, which supposedly allows them to cope better with the D_2O caused stress. In contrast, the increasing temperature increases the metabolic rates thus increasing growth rates and decreasing the deuterium caused stress. The cells at higher temperatures will grow and divide but to the similar final number of daughter cells. This suggests concentration and species-specific mechanisms governing the growth in D_2O, which are possibly related to a concentration of deuterated molecules within the cells. Furthermore, it implies that adjusting the metabolic rates to the deuterium concentration improves cell tolerance.

Based on our Raman microscopy measurements, when grown in D_2O, the first molecule to be deuterated is starch. This was also confirmed independently by nanoSIMS analysis. The deuteration of starch increases with the duration of cultivation and it becomes one of the most abundant molecules in such cells. This is because the photosynthesis is not significantly affected for a major part of the cultivation while cell division is slowed down and the number of formed daughter cells is lower than suggested by mother cell size. Thus, bigger daughter cells with more starch are formed by cell division, which is further exacerbated with each division. Our Raman spectroscopy data revealed high levels of deuteration of DNA for all three algae upon cultivation in D_2O. DNA extracted after DNA replication and cell division was deuterated by biosynthesis at otherwise hardly deuterable carbon positions of all four

bases. This implies the robustness of the storage and transfer of genetic information, which is not precluded by the pronounced deuteration of genetic material. Furthermore, our previous nanoSIMS results imply that limiting DNA deuteration migh improve cell tolerance.

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Effect of metals

The effect of different metals on algal physiology and possible biotechnological consequences has been one of the stable projects in the laboratory. We have closed our long-term study of selenium in algae summarizing the present knowledge and our findings in the review book chapter (Vítová et al., 2015). We have continued with the research of rare earth elements (REEs or lanthanides), their impact on algal physiology and recovery or recycling options.

The unique magnetic and catalytic properties predetermine REEs for exploitation in almost all electronic and clean energy technologies. In addition to the industrial sector, REEs are also utilized in agriculture as fertilizers, growth enhancers or in aquaculture. REEs are considered as critical raw materials because of their high supply risk and economic importance. With increasing demand, the requirement for recycling of REEs from industrial waste has risen. Research has recently focused on environmentally-friendly technologies of metal recovery from secondary resources including biological methods.

We followed up on our findings that under calcium dependent conditions REEs can replace calcium and thus improve algal survival. This suggests although they are non-essential, they 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 REEs on the algal growth and physiology as well as the possible exploitation of algae for REEs recycling or remediation has been discussed in our review (Goecke *et al.*, 2015b). We studied the effect of REEs on photosynthesis, growth, and chlorophyll profile of the model green alga *Desmodesmus quadricauda* (Řezanka et al., 2016) and the growth rate, lipidic, fatty acid and pigment profile in algae *Parachlorella kessleri* and *Trachydiscus minutus* (Goecke et al., 2017a). To follow the localization of single REEs in the algal cells by fluorescence microscopy, we developed the staining method using Fluo-4 probe. To include environmental aspects to our knowledge about REEs and algae, we conducted together with the Chilean team, the study of REEs distribution in macroalgae from different locations in Chile (Goecke et al., 2017b). Our knowledge about REEs and algae we summarized in the review (Vítová et al., 2019).

To evaluate the potential of microalgae for recycling or recovery of REEs from secondary resources, we designed series of experiments with different green algae and different e-waste sources of REEs including luminophores from fluorescence lamps or red mud, the by-product of alumina production (Čížková et al., 2018; Čížková et al., 2019a). We introduced a new model organism in our laboratory - the extremophilic unicellular red alga *Galdieria* serving for our applied research experiments and we developed a protocol for the cultivation (Čížková et al., 2019b;

Čížková et al., 2019c). We can consider the microalgae and especially the *Galdieria* as a good candidates for recovery of REEs from waste material.

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Lipidomics of microorganisms

Lipidomic studies of algae or other primarily extremophilic microorganisms as possible candidates for applications in biotechnology make a significant part of our research conducted in co-operation of the team of Dr. Řezanka (Pádrová et al., 2015; Vítová et al., 2016). The work includes identification of organisms producing odd chain and structured triacylglycerols (Řezanka et al., 2015a; Řezanka et al., 2015b) and very long chain fatty acids (Řezanka et al., 2018; Řezanka et al., 2019). We have also

studied the impact of polydatin on fatty acid metabolism in microorganisms (Řezanka et al., 2017), lipidomics of precursors of endocannabinoids in *Pectinatella magnifica* (Řezanka et al., 2018) and arsenolipids in the green alga *Coccomyxa* (Řezanka et al., 2019).

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Research activity and characterisation of the main scientific results

Publication output of the team significantly increased after the lab gained its full independence in 2015: from 17 publications over the period 2010-2014 to 37 publications during 2015-2019. The important trend is a higher contribution of our team in the individual studies, as well as their increasing quality.

In 2015 we published a review of Ecology of Anoxygenic Phototrophs in FEMS Microbiol. Rev. (IF~15) which currently represents the most authoritative summary of the field (Koblížek 2015). Our work on APB developed from mostly descriptive works we published before 2015, into studies focused more on the growth dynamics of APB. To accomplish that we used several unique and innovative approaches such as NIR epifluorescence microscopy (Ferrera et al. 2017), our own techniques of bacteriochlorophyll decay assessment (Cepáková et al. 2016) or gene expression activity assessment (Kolesár Fecskeová et al. 2019). Especially, the last study was technically very challenging since we attempted to analyse diversity of RNA transcripts in APB, which represents only a tiny fraction of the natural freshwater microbial communities.

We performed intense laboratory studies with various phototrophic species focused on how their photosynthetic apparatus is regulated under various physiology conditions (Piwosz et al. 2018, Kaftan et al 2019). The results of the laboratory experiments are important to understand APB physiology under real field conditions.

Our discovery of phototrophic Gemmatimonadetes made it into the new 15th edition of Brock Biology of Microorganisms (one paragraph based exclusively on our results). The fact that our work on phototrophic Gemmatimonadetes appeared in this internationally recognized Microbiology textbook already 3 years after its publication documents the importance of our work. As a new direction we commenced biochemical characterization of *G. phototrophica* photosynthetic complexes. Their preliminary characterization was published in Plos Biology (Dachev et al. 2017). Last year we obtained in collaboration with Dr. Pu Qian from Cambridge 3.4Å cryoEM structure of this complex which we currently prepare for publication.

A completely new direction represents our study of growth rates of freshwater bacterioplankton (Piwosz et al. ISME J, 2018). We developed a new methodology which utilize 16S amplicon sequences to reconstruct growth curves for all individual

bacterial OTUs (~400 OTUs). In this work was not focused on APB. He we tried to extend our activities to into general microbial ecology with much broader audience. The developed method has, we believe, its potential for many possible applications.

Selected publications of the team 2015-2019

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Ferrera I, Sánchez O, Kolářová E, Koblížek M, Gasol JM (2017) Light enhances the growth rates of natural populations of aerobic anoxygenic phototrophic bacteria. ISME J 11: 2391-2393.

Dachev M, Bína D, Sobotka R, Moravcová L, Gardian Z, Kaftan D, Šlouf V, Fuciman M, Polívka T, Koblížek M (2017) Unique double concentric ring organization of light harvesting complexes in *Gemmatimonas phototrophica*. PLOS Biology 15: e2003943.

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Piwosz K, Kaftan D, Dean J, Šetlík J, Koblížek M (2018) Nonlinear effect of irradiance on photoheterotrophic activity and growth of the aerobic anoxygenic phototrophic bacterium *Dinoroseobacter shibae*. Environ Microbiol 20: 724-733.

Piwosz K, Shabarova T, Tomasch J, Šimek K, Kopejtka K, Kahl S, Pieper DH, Koblížek M (2018) Determining lineage-specific bacterial growth curves with a novel approach based on amplicon reads normalization using internal standard (ARNIS). ISME J 12: 2640-2654.

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Piwosz K (2019) Weekly dynamics of abundance and size structure of specific nanophytoplankton lineages in coastal waters (Baltic Sea). Limnol Oceanogr 64: 2172-2186.

Kaftan D, Bína D, Koblížek M (2019) Temperature dependence of photosynthetic reaction centre activity in *Rhodospirillum rubrum*. Photosynth Res 142: 181-193.

Kolesár Fecskeová L, Piwosz K, Hanusová M, Nedoma J, Znachor P, Koblížek M (2019) Diel changes and diversity of *pufM* expression in freshwater communities of anoxygenic phototrophic bacteria. Sci Rep 9, 18766.

Research activity and characterisation of the main scientific results

The following text describes the main results achieved by our team during 2015–2019, based on 10 selected principal publications. The team's share on the creation as well as the significance of the respective publications are specified separately in the individual attached documents.

We continued our research regarding the biodegradation of environmental pollutants using a combination of approaches and methodologies, including microbiology, analytical chemistry, toxicology, and others. The mycobiota of a soil historically contaminated by **petroleum hydrocarbons** was characterized with subsequent isolation of filamentous fungal species. The most promising species, *Pseudoallescheria* sp. 18A, capable of utilizing the aliphatic hydrocarbons as the sole carbon source and successfully colonizing a lignocellulose substrate, was selected for a further bioaugmentation experiment in microcosms which resulted in 80% removal of the contaminants as well as a substantial decrease in toxicity. Furthermore, the mere amendment of sterile lignocellulose substrate lead to similar results by stimulating the autochthonous microflora suggesting that in this particular instance, a simple biostimulation can be used as a viable decontamination technique (Covino et al., 2015, *Science of the Total Environment* 505, 545–554).

The study of microbial consortia was also the objective of another publication dealing with the co-composting of **polycyclic aromatic hydrocarbons (PAHs)** from creosote-treated wood. In a pre-pilot-scale experiment (400 l), 97% of PAHs was removed after 240 days of composting with an amendment of grass cuttings (1:1 ratio). The degradation rate considerably exceeded the bioavailability of the detected PAHs which was estimated by supercritical fluid extraction. Furthermore, the removal of PAHs corresponded to a decrease in toxicity. Phospholipid fatty acid analysis and next-generation sequencing enabled for the first time a detailed characterization of taxa present during different phases of PAH co-composting. A rapid growth of microbial biomass was recorded in the early phases. The highest degradation rate was observed under thermophilic conditions dominated by Gram-positive prokaryotes (Bacilli and Actinobacteria) and ascomycetous yeasts (Saccharomycetales). During the course of composting, bacterial composition shifted in favour of Proteobacteria dominating in the later phases, including maturation (Covino et al., 2016, *Journal of Hazardous Materials* 301, 17–26).

Like PAHs, **polychlorinated biphenyls (PCBs)** are classified as persistent organic pollutants because of their recalcitrant character and belong among the most studied compounds in terms of (bio)remediation. An in-depth chemical and microbiological characterization of three layers (bulk, topsoil and rhizosphere) of a soil historically contaminated by PCBs was performed in order to assess possible biodegradation potential. Overall, the soil showed signs of ongoing natural attenuation (shifts in PCB congener composition, metabolite identification) with rhizosphere soil containing the least amount of PCBs. Proteobacteria dominated in all three soils and bacterial genera with known PCB degradation capabilities (e.g., *Rhodococcus*) were also detected, though their involvement in the degradation is theoretical. Fungi were mainly comprised of species belonging to the Basidiomycota and Ascomycota phyla (Stella et al., 2015, *Science of the Total Environment* 533, 177–186).

Our research team specializes in the study of biodegradation capabilities of **white-rot** (also referred to as **ligninolytic**) **fungi**. These model organisms produce extracellular enzymes (e.g., manganese-dependent peroxidase, lignin peroxidase, laccase) with low-substrate specificity that enable them to degrade the aromatic structures in lignin and consequently also a broad range of aromatic pollutants, including extremely recalcitrant compounds such as PCBs and polychlorinated dibenzo-*p*-dioxins. We continued with our research of **PCB degradation** by an assessment of the degradation potential of two representatives of white-rot fungi, *Pleurotus ostreatus* and *Irpex lacteus*, in real contaminated soil. The best degradation was observed with *P. ostreatus* in rhizosphere soil (50.5% after 12 weeks) and numerous transformation products were identified (hydroxylated and methoxylated PCBs, chlorobenzoic acids, chlorobenzaldehydes, chlorobenzyl alcohols). With the aid of next-generation sequencing and phospholipid fatty acid analysis it was found that *P. ostreatus* colonized the soil successfully and stimulated the growth of bacteria, particularly Firmicutes, while it supressed other fungal genera (Stella et al., 2017, *Journal of Hazardous Materials* 324, 701–710).

Apart from traditional persistent pollutants like PAHs and PCBs, our research is also focused on the detection, toxicological profiling and biodegradability assessment of new types of **micropollutants**, especially pharmaceuticals and personal care products.
For example, five ligninolytic fungi (*I. lacteus*, *Panus tigrinus*, *Dichomitus squalens*, *Trametes versicolor*, *P. ostreatus*) were screened for their ability to degrade **fluoroquinolone antibiotics** norfloxacin, ofloxacin and ciprofloxacin. *I. lacteus* and *T. versicolor* were identified as the most perspective degraders after reaching almost complete degradation after 10 and 14 days when grown in malt extract–glucose medium, respectively. Metabolite identification revealed similar mechanisms being employed in the transformation of these structurally similar compounds by all of the fungi. Principal component analysis was used to determine which reaction products likely participated in residual antibacterial activity detected after the degradation. Moreover, manganese-dependent peroxidase was proposed as the main degradative enzyme (Čvančarová et al., 2015, *Chemosphere* 136, 311–320).

A large number of both traditional pollutants and new types of micropollutants have been found to exhibit hormonal activity. The biodegradation capabilities of ligninolytic fungi towards some typical representatives of endocrine disruptors (EDs) – nonylphenols, bisphenol A, 17α-ethinylestradiol, PCBs – were reviewed with special attention being paid to particular intracellular and extracellular enzymes and mechanisms involved in the transformations (Cajthaml, 2015, *Environmental Microbiology* 17, 4822–4834).

Endocrine-disrupting compounds bisphenol A, estrone, 17β-estradiol, estriol, 17α-ethinylestradiol, triclosan and 4-*n*-nonylphenol were efficiently degraded in various types of fungal bioreactors (batch, trickle-bed). The bioreactors were filled with the so-called ‘spent’ substrate containing the mycelium of *P. ostreatus* HK35, a strain commonly used in oyster mushroom production. The spent substrate is a waste product of commercial mushroom cultivation and is therefore very inexpensive and readily available for mycoremediation applications. The reactor setup was initially tested at the laboratory scale and then was successfully scaled up and used to treat

real contaminated water from a wastewater treatment plant. The fungus was able to efficiently degrade EDs and simultaneously supress endocrine-disrupting activity even in an abundant presence of wastewater bacteria (Křesinová et al., 2018, *New Biotechnology* 43, 53–61).

Following incomplete removal during wastewater treatment processes, some extensively consumed **pharmaceuticals and personal care products** are continuously released into recipients and are being detected in trace amounts in wastewater, the environment and in some cases even in drinking water. This represents a potential problem not only for human health, but for aquatic organisms in particular, especially because information about their occurrence, toxicity and degradability is scarce. We have tested estrogenic and androgenic potencies of selected **pharmaceutical drugs** *in vitro* using the human T47D cell line and a luciferase assay using recombinant yeasts. Ibuprofen and diclofenac exhibited anti-estrogenic properties with both essays, while amiodarone only acted as an anti-estrogen with the T47D cell line. In addition, ketoprofen, naproxen and clofibrate, were shown to exhibit dose-dependent anti-androgenic and anti-estrogenic effects in the yeast-luciferase assays. On the contrary, other compounds (e.g., caffeine) did not show any hormonal activity in the two tests (Ezechiáš et al., 2016, *Chemosphere* 152, 284–291).
Similarly, hormonal activities of selected personal care products were also investigated. From this broad group of compounds, we focused on **additives and active compounds used in oral hygiene products** (toothpastes, mouthwashes) that are produced world-wide in ever-increasing quantities. We have tested their agonistic and antagonistic properties on human estrogen and androgen receptors using two human cell lines (T47D, AIZ-AR) and two recombinant strains of *Saccharomyces cerevisiae* (BMAEREluc/ERα, BMAEREluc/AR). None of the tested compounds acted as agonists of the respective receptors; however, octenidine and hexadecylpyridinium were revealed to be able to completely inhibit both estrogenic and androgenic signaling pathways. Even natural terpenes, thymol and menthol, were identified to exhibit anti-estrogenic and/or anti-androgenic effects, although the response was much weaker in comparison. In contrast, chlorhexidine was found to possess only anti-estrogenic properties (Michalíková et al., 2019, *Chemosphere* 217, 534–541).

Another part of our research interest involves the study of nanomaterials based on **zero-valent iron (nZVI)**. These materials are widely used in decontamination practice despite the fact that information about their toxicity is limited. This is because the specific properties of nZVI (e.g., high reactivity, coagulation, sorption on cells) interfere with the performance of common ecotoxicological assays. On that account, we have developed a new test assessing oxidative stress induced upon exposure to nanoparticles based on malondialdehyde quantification. The test was verified on six bacterial strains (*Escherichia coli* CCM 3988, *Bacillus subtilis* CCM1999, *Serratia marcescens* CCM 303, *Bacillus cereus* DBM 3035, *Staphylococcus epidermidis* DBM 3072, and *Enterobacter cloacae* DBM 3126) with 4 structurally different nanomaterials in environmentally relevant concentrations. The strains showed similar responses; total malondialdehyde production was correlated with the specific surface of the bacterial membrane. Malondialdehyde production was also influenced by the composition of the materials (connected to reactivity), confirming the suitability of the assay (Semerád et al., 2018, *Chemosphere*, 213, 568–577).

Research activity and characterisation of the main scientific results

Main task of the group is conducting world-class basic (fundamental, curiosity-driven) research on subjects defined by the principal investigators in third-party funded projects, publish the results in scientific literature (journals or books) and thus disperse the novel information among colleague researchers, students, and practitioners, eventually also advice policy makers and inform general public. To this end, the focus is slightly changing between years based on the third-party funding acquired. In the past, the group focus was quite dispersed among various topics but within the last 5 years, the momentum was gained by sharpened focus on plant-mycorrhiza associations, carbon for mineral nutrient trading between mycorrhizal partners, mycorrhizal hyphae-associated microbiome and mycorrhizal networks. This research has been supported by acquisition of specific equipment allowing state-of-the-arts research in this direction (isotope mass ratio spectrometer, IRMS, with a number of peripheries allowing analyses of gas, liquid and solid samples), and increased cooperation with on-campus molecular and NGS facilities (pyrosequencing, MiSeq, ultracentrifugation, Sanger sequencing, real-time PCR, microarray analysis infrastructure etc.). Novel methods are being adapted and/or developed in the group for quantitative real-time PCR assessment of large microbial groups and of specific functional microbial guilds in the soil and root samples, individual ecto- (e.g., truffle) and arbuscular mycorrhizal fungal species, and trophically linked microbes by using stable-isotope-probing approaches. These activities lead to number of publication outputs in international journals authored by the group members (usually 10+ papers/year), book chapters and conference contributions.
Major achievements of the past few years were published studies on the spatiotemporal dynamics of truffle hyphal colony in the fields, particularly with respect to patchiness of environmental conditions and associated microbiomes (Gryndler et al. 2015, 2017). Further, we published a series of studies quantifying carbon budgets of mycorrhizal plants as well as carbon-for-phosphorus rates of exchange in mycorrhizal symbiosis (Konvalinková et al. 2015, 2017, Řezáčová, Slavíková et al 2018). Last but not least, we recently published highly influential research on organic nitrogen – mycorrhiza – microbiome interactions, particularly with respect to competition between AM hyphae and ammonia oxidizers for free ammonia ions in soil (Bukovská et al. 2018).

Research activity and characterization of the main scientific results

Our laboratory is generally focused on various **molecular aspects of *Bordetella pertussis* pathogenicity**. *B. pertussis* is a re-emerging human pathogen of the respiratory tract and the etiological agent of whooping cough (pertussis). Despite vaccination programs, **pertussis remains one of the 10 most common causes of vaccine-preventable deaths** and pertussis incidence is currently on the rise in industrialized countries with highly vaccinated populations. We believe that the re-emergence of pertussis strongly suggests that we need to widen our understanding of the molecular mechanisms underlying the pathogenicity of *B. pertussis*. Therefore, we use genomics, transcriptomics, proteomics and a great variety of *in silico* tools to characterize the **evolution and adaptation of the global *B. pertussis* population**, to decipher molecular mechanisms involved in the **control of virulence and physiological fitness**, to analyze the **interplay between *B. pertussis* and human phagocytic cells** and to identify **novel biomarkers required for pertussis pathogenesis**. In our projects, we apply state-of-art omics techniques such as *de novo* genomic sequencing using Pac-Bio platform, comparative genomics using Illumina platform, DE RNA-seq, differential RNA-seq, dual RNA-seq and LC-MS/MS label-free proteomics. In detail, we study:

1. The link between changes in genomic, transcriptomic and proteomic profiles in Czech *B. pertussis* strains isolated in pre-vaccine and vaccine eras to identify the alterations in the genomic structures of Czech strains within the 60 years of vaccination and elucidate how these changes translated into gene expression and protein production profiles and, consequently, phenotypic diversity.

2. The role of RNA chaperone Hfq and small regulatory RNAs in virulence and fitness of *B. pertussis*.

3. The regulatory mechanisms governing the expression and production of T3SS components and elucidation of the role of T3SS in *B. pertussis* pathogenesis.

4. The interaction of *B. pertussis* with human macrophages with special focus on interplay between intracellular pathogen cells and host phagocytic cells during infection.

5. Expression and production of virulence and fitness factors under different environmental conditions (iron and glutamate limitation, blood induction, antigenic variation induced by modulators) and identification of novel biomarkers of *B. pertussis* pathogenicity.

Ad 1. We have compared genomic organization and transcriptomic and proteomic profiles of Czech clinical isolates collected between 2008 and 2015, Czech vaccine strains isolated from 1954 to 1965 and reference strain Tohama I. The SNP-based phylogenetic analysis of Czech strains and more than 350 complete *B. pertussis* genome sequences currently deposited in the GenBank showed that Czech isolates

cluster with isolates from other countries demonstrating world-wide spread and lack of geographical signature. Importantly, this result indicates that our data obtained with Czech isolates are applicable to strains from other countries. When compared to vaccine strains, genome size of Czech recent isolates was substantially reduced thereby confirming ongoing gene loss process within the global population of *B. pertussis*. The alignment of closed genomes **revealed that all genomes contain large-scale structural rearrangements** when compared to the reference strain Tohama I. According to their genome organization, sequenced strains could be classified into eight groups. The phylogenetic tree based on the genome organization of Czech strains demonstrated clear separation of historical and recent Czech isolates. Importantly, subsequent RNA-seq and LC-MS/MS analyses clearly indicated that **genomic variations translated into discretely separated transcriptomic and proteomic profiles**. Recent isolates displayed increased expression of flagellar genes and decreased expression of polysaccharide capsule operon compared to vaccine strains. Furthermore, compared to reference strain Tohama I, Czech strains exhibited increased expression of genes encoding Type III secretion system apparatus (T3SS). In spite of 50 years of vaccination, Czech vaccine strains differ from recent isolates to a lesser extent than from another vaccine strain Tohama I. Collectively, our data suggest that besides shaping the evolution of *B. pertussis* on a genomic scale, the **genome rearrangements affect also the global gene expression and proteomic profiles**. We assume that this mechanism counterbalances the low level of genetic variability observed in this pathogen and significantly contributes to adaptation of global population of *B. pertussis*.

Output:

Dienstbier A., Pouchnik D., Wildung M., Amman F., Hofacker I.L., Parkhill J., Holubova J., Sebo P., Vecerek B. Comparative genomics of Czech vaccine strains of Bordetella pertussis. Pathog Dis. 2018 10.1093/femspd/fty071.

We were responsible for concept and design of the study, strain cultivation, isolation of genomic DNA, genome structure- and SNP-based phylogenetic analysis, writing, correction and handling of the manuscript

Ad 2. The RNA chaperone Hfq and small regulatory RNAs are key factors in posttranscriptional regulation in bacteria and play an essential role in virulence of a broad spectrum of bacterial pathogens. We have shown that Tohama I Δ*hfq* strain produces decreased amounts of adenylate cyclase toxin and secreted reduced amounts of pertussis toxin. Likewise, expression of autotransporter *vag8* and tracheal colonization factor *tcfA* was strongly reduced in the *hfq* mutant. Consequently, **the *hfq* mutant was clearly attenuated in the mouse respiratory model of infection** as its lethality as well as capacity to colonize mouse lungs was strongly reduced when compared to the wt strain. In the follow up study we characterized the Hfq regulon in bacterial pathogen using an integrative omics approach. Gene expression profiles were analyzed by RNA-seq and protein amounts in cell-associated and cell-free fractions were determined by LC-MS/MS technique. Comparative analysis of transcriptomic and proteomic data revealed solid correlation considering the role of Hfq

in post-transcriptional control of gene expression. Importantly, our study confirmed and further enlightened the role of Hfq in pathogenicity of *B. pertussis* as it showed that **Δ*hfq* strain displays strongly impaired secretion of substrates of T3SS and substantially reduced resistance to serum killing**. On the other side, significantly increased production of proteins implicated in transport of important metabolites and essential nutrients observed in the mutant seems to compensate for the physiological defect introduced by the deletion of the *hfq* gene. The requirement for Hfq suggested that non-coding sRNA might be involved in regulation in *B. pertussis* virulence. Indeed, our analysis of the primary transcriptome of *B. pertussis* revealed hundreds of putative sRNAs and several of them were confirmed. One of them, RgtA, represents the **first fully characterized regulatory sRNA RgtA in *B. pertussis***. Abundance of this sRNA is strongly decreased in the absence of the Hfq protein and its expression is modulated by the activities of the two-component regulatory system BvgAS and another response regulator RisA. Furthermore, we identified plausible target gene of RgtA, the glutamate transporter, as well as the mechanism RgtA-dependent riboregulation. We propose that this sRNA is involved in control of transport of glutamate, an important source of carbon and upon infection it assist in adaptation of the pathogen to other sources of energy.

Output:

Bíbová I., Hot D., Keidel K., Amman F., Slupek S., Černý O., Gross R. and Vecerek B. Transcriptional profiling of Bordetella pertussis reveals requirement of RNA chaperone Hfq for Type III secretion system functionality. RNA Biology 2015, 12:175-185,
We were responsible for concept and design of the study, preparation of the samples (mice infection), downstream analyses (RT-qPCR, WB), writing and handling of the manuscript.

Amman F., D'Halluin A., Antoine R., Huot L., Bibova I., Keidel K., Slupek S., Bouquet P., Coutte L., Caboche S., Locht C., Vecerek B. Hot D. Primary transcriptome analysis reveals importance of IS elements for the shaping of the transcriptional landscape of Bordetella pertussis. RNA Biol. 2018, 15: 967–975
We were responsible for concept and design of the study, downstream analyses (RT-qPCR, Northern blot analysis), and writing of the manuscript.

Keidel K., Amman F., Bibova I., Drzmisek J., Benes V., Hot D., Vecerek B. Signal transduction-dependent small regulatory RNA is involved in glutamate metabolism of the human pathogen Bordetella pertussis. RNA 2018, 24: 1530-1541
We were fully responsible for the concept and design of the study, experiments (RNA-seq, Northern blot analysis, WB), and writing and handling of the manuscript.

Dienstbier A., Amman F., Stipl D., Petrackova D., Vecerek B. Comparative Integrated Omics Analysis of the Hfq Regulon in Bordetella pertussis. Int. J. Mol. Sci. 2019, 20(12). pii: E3073. doi: 10.3390/ijms20123073
We were fully responsible for the concept and design of the study, experiments (RNA-seq, proteomics), and writing and handling of the manuscript.

Ad 3. Comparative transcriptional profiling revealed that under laboratory conditions as well as upon passage in the host, the Hfq protein is required for expression of several virulence factors in *B. pertussis* cells including the T3SS. In striking contrast to the wt strain, T3SS did not become operational in the *hfq* mutant passaged either through mice or macrophages thereby proving that **Hfq is required for the functionality of the *B. pertussis* T3SS**. Furthermore, we have shown that similarly to isolates from other countries, Czech clinical isolates produced increased amounts of T3SS substrates. Finally, our recent experiments revealed that upon contact with blood, expression and secretion of T3SS components is strongly increased. In fact, when we compared cell-free fractions of *B. pertussis* cultures grown in the presence or absence of blood, we observed strongly increased amounts of T3SS substrates such as translocators BopB and BopD, effectors BopN and BopC and needle tip complex protein Bsp22 in the cultures treated with blood.

Output:
Bíbová I., Hot D., Keidel K., Amman F., Slupek S., Černý O., Gross R. and Vecerek B. Transcriptional profiling of Bordetella pertussis reveals requirement of RNA chaperone Hfq for Type III secretion system functionality. RNA Biology 2015, 12:175-185,
We were responsible for concept and design of the study, preparation of the samples (mice infection), downstream analyses (RT-qPCR, WB), writing and handling of the manuscript.

Ad 4. *B. pertussis* was historically described as an extracellular pathogen, which, in the presence of specific antibodies, can be internalized and killed by phagocytic cells. Nevertheless, some reports suggested that in the absence of opsonization, this pathogen stimulates its own attachment to immune cells resulting in inefficient killing by phagocytes and increased intracellular survival. These data led to speculations that *B. pertussis* can use macrophages as an intracellular niche and that the intramacrophage phase of infection could play a significant role in survival and persistence of bacteria within the host. To gain insight into *Bordetella*-macrophage interplay we used RNA sequencing to analyze the changes in gene expression profiles of human THP-1 macrophages resulting from *B. pertussis* infection. In parallel, we attempted to determine the changes in transcriptomic profiles of intracellular bacteria resulting from interaction with macrophages. Our analysis revealed that **global gene expression profiles in THP-1 macrophages are extensively rewired 6 h post infection**. Among the highly expressed genes we identified those encoding cytokines, chemokines and transcription regulators involved in the induction of the M1 and M2 macrophage polarization programs. Notably, several host genes involved in the control of apoptosis and inflammation which are known to be hijacked by intracellular bacterial pathogens were overexpressed upon infection. Furthermore, *in silico* analyses identified large temporal changes in expression of specific gene subsets involved in signaling and metabolic pathways.
When we analyzed bacterial response to internalization, we observed reduced expression of majority of virulence factors suggesting that **intracellular *B. pertussis* cells switch from virulent to avirulent phase**. On the other side, expression of

several genes involved in transport and metabolism of sulfate and several essential metals, redox homeostasis as well as of great variety of transcriptional regulators and factors was significantly increased. We assume that observed changes in the gene expression profiles indicate that **intracellular *B. pertussis* cells actively adapt to intramacrophage environment and respond to bactericidal activities triggered by THP-1 cells**. It seems that interplay between macrophages and intracellular *B. pertussis* cells may result in alternative polarization of macrophages and induction of the processes that augment survival of the bacteria within phagocytic cells.

Output:

Lamberti Y., Cafiero J.H., Surmann K., Valdez H., Holubova J., Vecerek B., Sebo P., Schmidt F., Völker U., Rodriguez M.E. Proteome analysis of Bordetella pertussis isolated from human macrophages. 2016, J. Proteomics 2016, 136:55-67
We have contributed to the concept of the study and correction of the manuscript.

Cafiero J.H., Lamberti Y.A., Surmann K., Vecerek B., Rodriguez M.E. A Bordetella pertussis MgtC homolog plays a role in the intracellular survival. PLoS One 2018, 10.1371/journal.pone.0203204
Substantial part of the work was designed and performed during stay of Mag. Cafiero in Prague.

Petrackova P., Farman M.R., Amman F., Linhartova I., Dienstbier A., Kumar D., Drzmisek J., Hofacker I., Rodriguez M.R. and Vecerek B. Transcriptional profiling of human macrophages during infection with Bordetella pertussis. RNA Biol. 2020, 10.1080/15476286.2018.1462655
We were responsible for the biological part of the study including development of the infection protocol, optimal differentiation of the macrophages, infection and RNA isolation, writing and handling of the manuscript.

Ad 5. Adaptability is one of the crucial features of microorganisms allowing them to endure and thrive within a wide range of environmental conditions. In order to adapt, bacteria must sense and respond to any alteration of the environment such as nutrient or oxygen limitation, pH and osmotic stresses and changes in temperature. Change in the ambient temperature is one of the major stresses that bacteria face as it, among other effects, influences the fluidity of their plasma membrane and function of the cell envelope. Recently, we have analyzed the thermal adaptation in two closely related respiratory pathogens *B. pertussis* and *B. bronchiseptica*. While *B. pertussis* represents a pathogen strictly adapted to the human body temperature, *B. bronchiseptica* causes infection in a broad range of animals and survives also outside of the host. In response to low temperatures, *B. pertussis* adapted its fatty acid composition and membrane fluidity to a considerably lesser extent when compared to *B. bronchiseptica*. Remarkably, ***B. pertussis* maintained the production of virulence factors at 24 °C** while *B. bronchiseptica* cells resumed the production only upon temperature upshift to 37 °C. This growth temperature-associated differential modulation of virulence factors production was linked to the phosphorylation state of transcriptional regulator BvgA. We proposed that the **reduced plasticity of the *B.***

***pertussis* membranes ensures sustained production of virulence factors at suboptimal temperatures and may play important role in the transmission of the disease**. Currently, we are determining the expression and production of important factors of pathogenesis under different nutrient stress conditions, such as iron and glutamate limitation.

Output:

Seydlova G., Beranova J., Bibova I., Dienstbier A., Drzmisek J., Masin J., Fiser R., Konopasek I., Vecerek B. The extent of the temperature-induced membrane remodeling in two closely related Bordetella species reflects their adaptation to diverse environmental niches. J. Biol. Chem. 2017, 292:8048-8058

This work was based on collaboration with the team of Dr. Konopasek from Charles University in Prague. Our contribution was significant (50%, corresponding author) and we were responsible for concept and design of the study, preparation of samples, downstream analyses (RT-qPCR, WB), and writing and handling of the manuscript.

Research activity and characterisation of the main scientific results

Microbiota and inflammatory diseases

To study the mucosal immunology and host-microbe interactions, we are focused mainly on the gut. Gut microbiota is a complex ecosystem consisting of vast number of bacteria, viruses, and fungi that have not been fully characterized yet. Numerous host-microbe interactions are shaping the microbial community and influence host's physiological functions, development of the immune system, and provoke various pathological conditions. Many multifactorial disorders occur as consequence of the disturbed mucosal barrier or as the results of alterations in the immune response to the components of gut microbiota. Therefore, by shaping the gut microbiota by diet, drugs or disease itself the individual may become more susceptible to the immune-mediated diseases such as inflammatory or autoimmune diseases or cancer. We study this interaction to gain deeper insight of the underlying mechanisms. By combining the approaches of basic research with analysis of samples from patients, we try to translate this knowledge into clinical practice. The effect of gut microbiota is not limited only to typical diseases of the gut, such as inflammatory bowel disease, but it may influence the skin inflammation during psoriasis, lung inflammation in asthma or eye inflammation in autoimmune uveitis. Here, we study which microbes are associated with these diseases and their complications and how these microbes interact with the host in order to improve the diagnostic, therapy or even prevention.

Biomarkers of human diseases

Antibody response against antigens and self-antigens is an integral part of adaptive immune mechanisms. However, the somatic effect of generated antibodies is pleiotropic, depending on their heterogeneity, i.e. the ratio of isotypes, specificity and affinity. In consistence with this fact, the role of various antibodies and autoantibodies is a matter of contention due to the absence of a clinical correlate. Nevertheless, the occurrence of certain serum antibodies is of diagnostic value. Notably, the testing of specific antibody may possess a promising prognostic or predictive potential. For this reason, in cooperation with physicians, we focused on the study of antibody response against gliadins and other food-proteins and autoantibodies in patients with active celiac disease, those on a gluten-free diet, patients with autoimmune disease associated with celiac disease, and in general population. Moreover, we also studied the occurrence of autoantibodies against the multifunctional protein calreticulin, their heterogeneity and fine specificity and clinical relevance in patients with autoimmune and oncological diseases and idiopathic cardiomyopathies (Hoffmanová *et al.: Physiol. Res.* **64:** 537-546, 2015; Sánchez *et al.: Autoimmunity* **49:** 554-562, 2016). The original findings of our studies are disseminated via a teaching activity (Theory of immunology methods; Faculty of Science, Charles University in Prague).

Next, we linked our interest in early diagnostics with our long-time passion for host-microbe interaction and start to study the predictive capacity of antimicrobial response and biomarkers of gut damage in multiple human diseases. We focused on intestinal fatty acid binding protein (I-FABP), which is released to the bloodstream, and later filtered to the urine, as a result of gut epithelium damage. We found that analysis of urinary I-FABP could not only predict the post-operative neonatal necrotizing enterologitis (NEC), but it can also distinguish it from sepsis (Coufal *et al.: J. Immuno.*

Res. **2016:** 5727312, 2016). We continued our research with other neonatal emergencies finding that while I-FABP is a marker for intestinal mucosa damage in gastroschisis, it fails to predict early recovery, so it is not suitable for outcome prediction in clinical settings (Kokesova *et al.: PLoS ONE* **14:** e0210797, 2019). Next, to gain an insight into the inflammatory bowel disease (IBD) pathogenesis, we analyzed serum biomarkers and specific anti-microbial B and T cell responses to the gut commensals in different forms of IBD. We found that decrease in matrix metalloproteinase (MMP)-9 and increase in MMP-14 are the strongest factors discriminating IBD patients from healthy subjects and that low transforming growth factor-β1 (TGF-β1) is associated with disease relapse and low osteoprotegerin with anti-tumor necrosis factor-alpha (TNF-α) therapy. Patients with CD have significantly decreased antibody and increased T cell response mainly to genera *Eubacterium*, *Faecalibacterium* and *Bacteroides*. These results stress the importance of the gut barrier function and immune response to commensal bacteria and point at the specific differences in pathogenesis of different forms of IBD (Coufal *et al.: Cells* **8:** 719, 2019).

Comparative immunology

To study the comparative and developmental immunity, we use quite unusual model organism, earthworm *Eisenia andrei*, which lives mostly in compost representing the environment with very high antigenic pressure. In contrast to sophisticated vertebrate or mammalian immune system, the use of invertebrate organism is, due to its simplicity, appropriate for the study of various aspects of innate immunity. Since invertebrates do not possess the acquired immunity, earthworms rely solely on the innate immunity in their defense strategies. The coelomic fluid of earthworms contains a great variety of molecules involved in direct elimination of invading microorganisms. We have identified several of such molecules, e.g. pattern recognition receptors – coelomic cytolytic factor, two Toll-like receptors and LBP/BPI molecule (Škanta *et al.: Dev. Comp. Immunol.* **54:** 1-6, 2016; Procházková *et al.: Front. Immunol.* **10:** 1277, 2019). These receptors recognize various conserved molecular structures of microorganisms. The highest abundancy of these molecules is present in the intestine representing the tissue in direct contact with microorganisms.

Earthworms participate in soil fertility. They are in a very close contact with the soil via both alimentary tract and highly permeable skin. They have been described to bioaccumulate various organic and inorganic pollutants and thus allow these substances to enter the food chain. For these reasons, earthworms are often used in monitoring soil ecotoxicity. In our laboratory, we follow the defense response of earthworms on various organic pollutants and also nanoparticles (Procházková *et al.: Env. Sci. Poll. Res.* **25:** 26267-26278, 2018; Roubalová *et al.: Ecotox. Environ. Safety* **159:** 363-371, 2018; Roubalová *et al. Env. Sci. Poll. Res.* **27:** 33429-33437, 2019).

Research activity and characterisation of the main scientific results

One of our research activities was focused on the study of biological effects of HPMA copolymer-based drug delivery systems bearing cytostatic drugs. We studied the relation of the structure/physico-chemical parameters and biological features/anticancer activity of the polymer drug delivery systems. Further, we investigated the possibility to augment the therapeutic activity of polymer-bound drugs via modulation of EPR effect, or by construction of polymer carriers with specific functions. We also evaluated the effect of the polymer-bound cytotoxic drugs on the antitumor immunity.

One of our project was aimed to compared linear (Mw ~27 kDa, Rh ~4 nm) and non-degradable star (Mw ~250 kDa, Rh ~13 nm) HPMA copolymer conjugates bearing doxorubicin bound via pH-sensitive hydrazone bond. We determined the *in vitro* and *in vivo* toxicity of both conjugates and their maximum tolerated dose (MTD). We also compared their antitumor activity in mouse B-cell leukemia BCL1 and a mouse T-cell lymphoma EL4 model. We found that MTD was higher for the linear conjugate (85 mg DOX/kg) and lower for the star conjugate (22.5 mg DOX/kg). An evaluation of the intestinal barrier integrity using FITC-dextran as a gut permeability tracer proved that no pathology was caused by the MTD of either conjugate. However, free doxorubicin showed some damage to the gut barrier. The therapy of BCL1 leukemia by both of the polymeric conjugates using the MTD or its fraction (i.e., equitoxic dosage) showed better results in the case of the star conjugate. On the other hand, treatment of EL4 lymphoma seemed to be more efficient when the linear conjugate was used. We suppose that the treatment of solid tumours and leukemias requires different types of polymer-drug conjugates. We hypothesise that the most suitable HPMA copolymer-bound doxorubicin conjugate for the treatment of solid tumors should have high molecular weight structure that would be stable for three to four days after the conjugate administration and then rapidly disintegrate in the short polymer chains, which are excretable from the body by glomerular filtration. On the other hand, the treatment of leukemia requires a polymer-drug conjugate with very long circulation half-life. This would provide an active drug, whilst slowly degrading to excretable fragments (Tomalova et al., *Journal of Controlled Release*, 223: 1-10, 2016).

We have shown in our other work that structure of the polymer carrier of cytotoxic drugs doxorubicin and docetaxel is critical in combination treatment. Only linear conjugates carrying both doxorubicin and docetaxel showed additive antitumor effect while star structure of polymer carrier showed subtractive effects upon combination of these two cytostatic drugs. The mechanism behind that is different pharmacokinetics of the drugs mediated by linear and star polymer conjugates, respectively (Sirova et al., *Journal of Controlled Release*, 246: 1–11, 2017).

The driving force of the drug accumulation in the solid tumors is EPR effect. Micellar polymer carrier bearing doxorubicin bound through hydrazone bond enables high accumulation of the drug in solid tumors. However, the polymer carrier needs to be able to disintegrate into smaller polymers capable of elimination from the body via kidney filtration. Three types of polymer micelles with various bloodstream stability were tested in respect to their cytotoxic and therapeutic activity. We have clearly shown that polymer micelles with highest stability had longest half-life in circulation accompanied by the highest doxorubicin accumulation in the tumor and superior therapeutic activity (Chytil et al., *Molecular Pharmaceutics*, 15: 3654−3663, 2018).

We have explored the possibility to enhance EPR effect in order to increase the accumulation of polymer-bound drugs in the solid tumor focus as even a little changes in their accumulation could result in a significant increase of their therapeutic activity. Polymer donors of nitric oxide (NO) were designed and tested as locally functioning vasodilators which could facilitate higher accumulation of polymer-bound drugs within the tumor via enhancement of the EPR effect. Our data showed that polymer NO donors are capable to increase the accumulation of polymer-bound doxorubicin in solid tumors and provide significant improvement of their antitumor activity (Studenovsky et al., *Journal of Controlled Release*, 269: 214-224–11, 2018).

A broad study of polymer carrier of cytotoxic drug with ability to overcome P-gp-mediated multidrug drug resistance of tumor cells was the subject of another project. Here we have shown that the diblock polymer carrier consisting of hydrophobic block based on poly(propylene oxide) and hydrophilic block based on poly(HPMA-co-Ma-AH-NHNH-Boc)-TT terminated with TT functional group (PPO-HPMA) forms micelles and could be used as a polymer drug delivery system. It accumulates in solid tumors via EPR effect and is capable to overcome the P-gp mediated multidrug resistance of tumor cells. PPO-HPMA diblock carrier-bound doxorubicin has very promising therapeutic activity in experimental tumor model with naturally elevated P-gp expression such as murine CT26 colon carcinoma. Moreover, structure and PPO content in the carrier (either part of the polymer diblock forming the micelles or entrapped in the micellar core by physico-chemical interaction) were studied as the most critical parameters affecting the antitumor capacity of the PPO-HPMA-based drug delivery systems (Braunova et al., *Journal of Controlled Release*, 245: 41–51, 2017; Braunova et al., *Pharmaceutics*, 11, 2019 doi:10.3390/pharmaceutics11110579).

One of our other project was aimed to overcome multidrug resistance of cancer cells via the use of combination of cytostatic drug and low molecular weight P-gp inhibitor. Here, we used HPMA copolymer conjugates, whereby the cytostatic drug doxorubicin or the derivative of the P-gp inhibitor reversin 121 (R121) or both were covalently bound through a degradable pH-sensitive hydrazone bond. We proved that R121, when bound to a polymeric carrier, is capable of inhibiting P-gp in P388/ MDR cells and sensitizing them in relation to the cytostatic activity of doxorubicin. Conjugate bearing both doxorubicin and R121 was found to be far more potent in P388/MDR cells than conjugate bearing doxorubicin alone or a mixture of conjugates bearing either doxorubicin or R121 when cytostatic activity *in vitro*, cell cycle arrest, accumulation of doxorubicin in cells and induction of apoptosis were determined. Importantly, conjugate bearing R121 was also effective *in vivo* as it inhibited P-gp in P388/MDR tumors after intraperitoneal administration, while both the conjugate bearing doxorubicin and R121 induced apoptosis in P388/MDR tumors more effectively than conjugate bearing doxorubicin alone. Only conjugate bearing doxorubicin and R121 significantly inhibited P388/ MDR tumor growth and led to the prolonged survival of treated mice. However, the most dramatic antitumor activity of this conjugate was found in the CT26 tumor model where it completely cured six out of eight experimental mice, while conjugate bearing doxorubicin alone cured no mice (Sivak et al., *Biomaterials*, 115: 65-80, 2017).

We also studied star biodegradable polymer systems based on 2,2-bis(hydroxymethyl)propionic acid (bisMPA) dendrons or dendrimers. The dendron/dendrimer core is grafted with monodispersed semitelechelic linear poly(HPMA) chains, to which the drug can be attached via suitable biodegradable bond. The hydrodynamic diameter of the star copolymer carriers can be adjusted to a given purpose by proper selection of the bisMPA dendritic core type, by considering the linear HPMA copolymer molecular weight and by polymer-to-core molar ratio.

Spontaneous hydrolytic degradation of the dendron or dendrimer core in the organism results in degradation of the polymer carries within several days. Thus, increased accumulation of the drug in the solid tumor tissue can be achieved together with elimination of the polymer from the body via renal filtration. Importantly, these biodegradable systems also showed superior therapeutic efficacy in a murine tumor model (Kostka et al., *Biomaterials*, 235, 2020, doi.org/10.1016/j.biomaterials.2019.119728).

The polymer-bound drug conjugates are characterized by EPR effect and prolonged pharmacokinetics of the carried drug which is different from that of the parent low-molecular weight drugs. This leads to high accumulation of the polymer-bound drug in the target tumor tissue as well as to a decreased exposure of normal tissues and cells to the cytotoxic drug resulting in reduction of systemic toxicity and lower harm to the immune system. This is significant advantage of the polymer-bound drugs. However, a number of immune suppressive mechanisms still operate in the solid tumors. They interferes with successful therapy which needs effective antitumor immune mechanisms. Two polymer conjugates carrying drugs with the capacity to modulate the immunosuppressive functions of myeloid-derived suppressor cells (MDSCs) were tested, namely all-trans retinoid acid (ATRA), and cucurbitacin D. These two drugs mediated MDSC-inhibition and showed some potentiation of the treatment efficacy of polymer-bound doxorubicin conjugates. Manuscripts summarizing this data are in preparation and they will be submitted in 2020.

Other research activity of our team was focused on biological features and potential therapeutic use of IL-2 complexes based on S4B6 and JES6.1 mAbs as well as elucidating the mechanism of mechanism of their action. We participated (in collaboration with laboratory of Christopher Garcia at Stanford University) in describing the detailed molecular structure of IL-2/JES6.1 mAb and IL-2/S4B6 mAb which led to understanding the mechanism how these IL-2 complexes interact with IL-2 receptor. This work showed that binding site of CD25 and S4B6 mAb in IL-2 molecule almost completely overlaps explaining why CD25 expression is irrelevant for utilization of IL-2/S4B6 mAb complexes. Contrary to JES6.1 mAb, S4B6 mAb does not impede binding of IL-2 to CD122 and CD132. Thus, IL-2/S4B6 mAb complexes can bind to dimeric intermediate-affinity IL-2 receptor and activate it. On the other hand, binding sites of JES6.1 mAb and CD122 as well as CD132 in IL-2 molecule significantly overlap and IL-2/JES6.1 mAb complexes are thus unable to bind to and activate IL-2 receptor directly. Although there is no steric clash between JES6.1 mAb and CD25, key residues in IL-2 molecule that contact CD25 change conformations upon binding to JES6.1 mAb. Thus, JES6.1 mAb binding to one site of IL-2 allosterically impairs CD25 binding to a non-overlapping site and this impaired binding (i.e., lowered affinity for CD25) renders JES6.1-bound IL-2 selective for $CD25^{high}$ cells which express sufficient CD25 levels to displace the JES6.1 mAb by mass action and initiate the „triggered exchange“ mechanism. CD25-bound IL-2 can consequently bind to CD122/CD132 dimer and initiate signaling downstream of IL-2 receptor (Spangler et al., *Immunity*, 42: 815–825, 2015).

The above described findings in addition to our expertise in designing immunocytokines (i.e. recombinant covalently linked cytokine/mAb constructs, ICs) led to ongoing project with our collaborating lab in the USA. We have designed, produced and characterized biological activity of JY3 chimeric protein where light chain of JES6.1 mAb is linked through flexible oligopeptide linker $(Gly_4Ser)_2$ to IL-2. Moreover, JY3 is IC with two point mutations (Y43A and Y101A) in light chain of the JES6.1 mAb leading to weakened affinity for IL-2. This lower affinity for IL-2 was found to be required for

exchange mechanism to work and thus to manifest biological activity of the resultant IC. We evaluated biological activity of JY3 in comparison to IL-2/JES6.1 mAb complexes (both commercial and recombinant mAb-based) and IL-2 mixed with irrelevant mAb. Further, we have validated our data by determination of Treg cell expansion and analyzing Foxp3 transcription factor and CD25 expression in these cells. We also analyzed expansion of $CD8^+$ T cells from OT-I transgenic mice adoptively transferred into congeneic mice and activated via SIINFEKL peptide administration and treated by high dose of JY3. Finally, we also determined the potential of JY3 in DSS-induced colitis model in mice. Altogether, we showed that treatment with JY3 IC is superior to IL-2/JES6-1 complexes in expansion of immune cell populations and prevention of experimental autoimmune disease (Spangler et al., *Journal of Immunology*, 201:2094-2106, 2018).

We have also published a review article summarizing the knowledge on biological activities and mechanism of action of IL-2 complexes and discussing potential clinical use of IL-2 complexes reflecting our expertise in this field (Tomala and Kovar, *OncoImmunology*, 5(3): e1102829, 2016).

Our collaboration with biotechnology company SOTIO dates back into 2014. Our first project was focused on evaluation of high hydrostatic pressure (HHP) effect on cancer cells. We found that HHP promotes key characteristics of immunogenic cell death (ICD), in thus far resembling immunogenic chemotherapy and ionizing irradiation. We demonstrated that cancer cells succumbing to HHP induce $CD4^+$ and $CD8^+$ T cell-dependent protective immunity *in vivo*. Moreover, we showed that cell death induction by HHP relies on the overproduction of reactive oxygen species (ROS), causing rapid establishment of the integrated stress response, eIF2a phosphorylation by PERK, and sequential caspase-2, 8 and 3 activation. Our results indicate that the ROS – PERK - eIF2a - caspase-2 signalling pathway is central for the perception of HHP-driven cell death as immunogenic (Moserova et al., *OncoImmunology*, 6(1): e1258505, 2017).

Second project in collaboration with SOTIO was similar to the first one. We studied the effect of mild heat-shock (mHS) treatment (< 42 ºC) and severe cytotoxic heat-shock treatment (sHS > 43 ºC) on tumor cells. Here, we found that sHS, but not mHS, induces immunogenic cell death in human cancer cell lines. sHS-treated murine cells exposed calreticulin, HSP70 and HSP90 and activated higher DC maturation than mHS treated cells. Vaccination with sHS-treated tumor cells elicited protective immunity in mice. In this study, we defined specific conditions for the sHS treatment of human lung and ovarian tumor cells to arrive at optimal ratio between effective cell death, immunogenicity and content of tumor antigens for immunotherapeutic vaccine generation (Adkins et al., *OncoImmunology*, 6(5): e1311433, 2017).

Our other collaboration with SOTIO was focused to study biological activities of a specific immunotherapeutic agent. The anti-tumor activity, pharmacodynamic and pharmacokinetic activities were investigated using several cancer models. The specific results are confidential.

Research activity and characterisation of the main scientific results

The research group under the leadership of **Marek Sinkora** is primarily concentrated to study the B and T lymphocyte development in swine. Pigs were historically grouped to animals that use 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. The results disproved 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; the paradigm that was maintained for over 30 years in other scientific reports, reviews, and immunology textbooks. Following of this 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., Dev Comp Immunol. 51:99, 2015). Accordingly, we have also showed that IPP) are the major source of primary, undiversified IgA antibodies in newborn piglets (Butler et al., Dev Comp Immunol. 65:340, 2016).

● On the other hand, using flow cytometry sorting and detection of rearrangement-specific transcripts and DNA products we showed that a major B lymphopoietic organ of pigs is the bone marrow. As a side effect of studies we have also shown that immunoglobulin light chain precedes heavy chain gene rearrangement during development of B cells in swine (Sinkora et al., J Immunol. 198:1543, 2017). To our knowledge, this is the first evidence that some mammals can use an inverted order of Ig loci rearrangement. These results may have important consequence for comparative immunology because it appears that there are two groups of animals, one of which uses a pre-BCR–driven developmental pathway for B cell generation whereas the second group uses a pre-BCR–independent pathway. Moreover, our results also explain the extreme deviations in the immunoglobulin light chain type ratios among mammals. All these aspects were also discussed and reviewed separately in two independent reports (Sinkora and Butler, Dev Comp Immunol. 58:1, 2016 and Butler et al., Annu Rev Anim Biosci. 5:255, 2017).

● Our studies of lymphocyte development also involve characterization of rearranged immunoglobulin heavy chain genes in thymus (Sinkorova et al., Dev Comp Immunol. 99:103396, 2019). We found three independent populations of cells: The first population can be found exclusively in medulla and it consists of existing mature B cells and plasma cells. The second consists of developing B cells characterized by the presence of selected rearrangement, similar to B cell lymphogenesis in the bone marrow. The third population is entirely unaffected by selection mechanism and represents T lineage cells that rearrange immunoglobulin genes. As far as we know, this is the first evidence that some species completely rearrange immunoglobulin genes in T cells. Our results also support the finding that B cells actively develop in the thymus, and these thymic B cells are a separate and non-recirculating population harboring a distinct function.

● Another element of research under the leadership of Marek Sinkora was concentrated to investigate the pathological immune response to porcine reproductive and respiratory syndrome virus (PRRSV), which is a major threat to swine health and

global pork production. According to our earlier results done before 2015, we have collected increasing evidence that immune dysregulation caused by PRRSV is due to infection of the thymus. We found that PRRSV interact with developing T cells and causes an acute deficiency that produces “holes” in the T cell repertoire allowing for poor recognition of PRRSV and other neonatal pathogens (Butler et al., Front Immunol. 10:1077, 2019). Currently, our newest project granted exclusively to our Laboratory is going to resolve other consequences of PRRSV infection in porcine thymus and explain mechanism by which PRRSV causes immune dysregulation.

●Last topic of our research was done in cooperation with the Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Libechov, Czech Republic. Using a porcine model, we describe Melanoma-Associated T-lymphocytes that increase during melanoma regression, and which have their direct counterparts in Tumor Infiltrating Lymphocytes (Cizkova et al., Dev Comp Immunol. 92:60, 2019). Both populations have a similar expression of selected markers indicating that the composition is identical. Moreover, our laboratory proved that these cells have mono-specific T-cell receptor that was further analyzed by sequencing. These results indicate that pigs regressing melanomas possess a characteristic population of recirculating T-cells playing a role in tumor control and regression. We contributed on this initial study by about 25% but our new project and interest is going to target the investigation of other aspects primarily to our Laboratory.

Part of the laboratory under leadership of **Martin Schwarzer:** During 2015-2019 reporting period Dr. Kozakova stepped down as a leader of a scientific group and a call was issued for a new group leader. Dr. Schwarzer returned to the Laboratory of Gnotobiology after his 3 year postdoc stay at the Institute of Functional Genomics in Lyon, France (9/2014 – 8/2017) and was selected as a best candidate for the new group leader. On the 1.1.2018 the Integrative Physiology of Gnotobionts group was established. There is a continuation in the research of the role microbiome, selected bacteria and their defined immunomodulatory components on the allergic sensitization and allergy development. Next to this, the group is investing heavily into understanding the enigmatic bacteria-host cross-talk in postnatal growth under chronic undernutrition. The central model of the group is gnotobiotic mouse.

● We finished our studies about the immunomodulatory properties of different *B. longum* ssp. *longum* (Bl) bacterial strains. We have shown that two different isolates of the same bacterial species Bl differ profoundly in their immunomodulatory capacities. Bl 7952 strain, but not Bl 372 strain, protected mice from the development of experimental colitis by reducing clinical symptoms and preserving expression of tight junction proteins resulting in improved intestinal barrier function. Our data suggest that although some immunomodulatory properties might be widespread among the genus *Bifidobacterium*, others may be rare and characteristic only for a specific strain. Therefore, careful selection might be crucial in providing beneficial outcome in clinical trials with probiotics in IBD (Srutkova et al., PlosOne 10(7), 2015).

● In collaboration with the group of Prof. Andrzej Gamian and later with the group of Dr. Sabina Gorska (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Department of Immunology of Infectious Diseases, Polish Academy of Sciences, Wroclaw) we continued our research about immunomodulatory properties

of bacterial cell wall derived antigens and their ability to modulate the allergic sensitization in a mouse model. We identified the immunoreactive proteins from two different isolates of *Bifidobacterium longum* ssp *longum* that reacted with gnotobiotic mono-colonized mouse sera, immune rabbit sera and non-immune human sera (Gorska et al., Front Microbiol. 7:1537, 2016). Next we identified the chemical structure and immunomodulatory properties of of polysaccharides isolated from probiotic *Lactobacillus casei* LOCK 0919 (Gorska et al., Glycobiology 26:1014, 2016). In these studies we provided the bacteria, monocolonized mouse sera and performed the *in vitro* immunomodulatory assessment of the isolated and characterized antigens. Next, continuing on our previous research on in vitro immunomodulatory capacity of two polysaccharides L900/2 and L900/3 isolated from *Lactobacillus rhamnosus* LOCK 0900 we showed that they are able to modulate allergic sensitization to ovalbumin (OVA) in a mouse model when administered together with the antigen. Our findings support and expand on our previous *in vitro* studies by demonstrating that polymer L900/3 might modulate the Th1/Th2 balance and could be a promising candidate molecule for preventing allergic sensitization. All the *in vivo* experiments related to the last study were performed in Laboratory of Gnotobiology by Dr. Gorska under our supervision (Gorska et al., Microb. Biotechnol. 10:586, 2017).

● In collaboration with Dr. Schabussova (Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University Vienna, Austria) we selected and characterized strain specific immunomodulatory properties of 3 different lactobacilli strains. We showed that colonization of germ-free mice with a mixture of these Lactobacillus strains improves the gut intestinal barrier and significantly lowers levels of allergen-specific antibodies after allergic sensitization (Kozakova et al., Cell Mol Immunol 13:251, 2016). Next we continued our work with OVA-induced food allergy (Golias et al., Plos One 7, 2012) mouse model. We transferred this model into the germ-free conditions. Systemic sensitization and oral challenge of GF mice with ovalbumin led to increased levels of specific IgE in serum compared to CV mice. Surprisingly, despite the high levels of sensitization, GF mice did not develop diarrhea or anaphylactic hypothermia, common symptoms of food allergy. We demonstrated that this was due to the lower maturation and defective gut-homing of mast cells in GF animals. We concluded that microbiota-induced maturation and gut-homing of mast cells is a critical step for the development of symptoms of experimental food allergy. This new mechanistic insight into microbiota-mast cells-food allergy axis can be exploited in the prevention and treatment of food allergy in humans (Schwarzer et al., Front Immunol. 10:205, 2019). In the collaboration with another group from Medical University of Vienna under the leadership of Dr. Maria Sibilia we performed key experiments on germ-free mice confirming that commensal skin microbiota provoke atopic-like inflammatory skin diseases by invading into the follicular opening of erupting hair. These findings can be extrapolated to atopic dermatitis in humans, improving our understanding of disease ontogeny and therapeutic possibilities by highlighting barrier defects as the initiating event (Klufa et al., Sci. Transl. Med. 11, 2019)

● We continued our research to decipher how microbiota and selected lactobacilli strains impact the postnatal growth of juvenile mice upon undernutrition and under the normal mouse diet régime. We wanted to find answers to these two questions: 1) What is the contribution of the gut microbiota to normal postnatal growth? and 2) How does it influence the activity of the somatotropic axis during feeding on rich diet and during chronic undernutrition? To explore the first question we utilized two groups of mice: normal (conventional) mice harboring the intestinal microbiota and germ-free mice, which are devoid of any detectable living bacteria. Juvenile male mice were weaned on the rich diet, which had all the necessary nutrients in sufficient amounts, and we followed their longitudinal and ponderal growth until young adulthood. Conventional mice showed better growth rate and their final weight and length was bigger compared to the germ-free counterparts, and such difference was not caused by bigger fat stores but overall organ and bone growth. Besides the macroscopic phenotype difference, we also found that microbiota improved the function of the somatotropic axis with higher titers of growth promoting IGF-1 in the sera of conventional mice. To prove that indeed the higher levels of IGF-1 were sufficient for the observed increased growth, we injected the germ-free mice with IGF-1 and their juvenile growth rate was improved. Having established the necessary role of microbiota in optimal growth in nutrient rich conditions, we wanted to know if it plays the same role in chronic undernutrition. To test this, we designed a diet, which was low in proteins, fats and vitamins, but provided the mice with the same amount of calories thanks to addition of carbohydrates. When we weaned the juvenile male mice on this depleted diet, we observed that the germ-free mice fail to grow in terms of weight and length – they were completely stunted. On the other hand, their conventional counterparts harboring the bacteria resume growing, although slower than the conventional mice fed the nutritionally rich diet. And again we could match this growth with better function of somatotropic axis and higher levels of IGF-1 in sera. Concerning the host growth and bacteria colonization, it has been previously shown that certain specific bacteria from the species *Lactobacillus (L.) plantarum* are able to promote growth of the Drosophila larvae upon nutrient scarcity (Storelli et al., Cell Metabolism 14:403, 2011). *L. plantarum* belongs to the lactic acid-producing bacteria and these are nomadic bacteria that can be found in different habitats, such as vegetables, soil and fermented foods, but also in the intestinal tracts of both invertebrates and vertebrates, including mice and humans. We were therefore wondering: Will the single bacterium which promotes growth in Drosophila model, be able to promote the growth of the stunted germ-free mice? And to what extent? To this end we mono-associated the germ-free mice with the growth-promoting strain L. plantarum WJL and submitted them to the depleted diet at weaning. We observed that the mono-associated mice grew as well as the conventional mice on the depleted diet and that this one *L. plantarum* strain improved the function of somatotropic axis to the same extent as we observed in the conventional mice. Our work established that microbiota is necessary and certain selected *L. plantarum* bacteria are sufficient to set on the somatotropic axis activity to boost juvenile growth rate. All the in vivo experiments using germ-free, gnotobiotic and conventional animals were performed in our Laboratory of Gnotobiology. The molecular characterization has been performed in collaboration with our partners in France (Schwarzer et al., Science 351:854, 2016). Further, we have reviewed the evidence about microbiota, selected bacterial strains and host growth in several papers (Schwarzer et al., Calcif. Tissue Int. 102:387, 2018; Schwarzer, Curr Opin Clin Nutr Metab Care 21:179, 2018; Poinsot et al., J Mol Endocrinol. 61:T103, 2018). The work in the laboratory continues and will continue to

decipher the underlying host-bacteria molecular dialogue behind the observed growth promotion phenomenon.

Part of the laboratory under leadership of **Tomas Hudcovic** using gnotobiotic mice models has mainly concentrated to study effects of microbiota on the development of experimentally induced human inflammatory diseases (IBD). We studied effects of component of commensal bacteria in pharmacokinetics of anti-inflammatory drugs. The activity of the laboratory was involved in the investigation of gut microbiota and neuroendocrine regulatory pathways during stress.

● 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 (DSS). 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 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 (Du et al., Gut Pathog. 7:32, 2015). In cooperation with Prof. J. Wells from Wageningen University, Netherlands, we participated on investigation of the role bacteria in IBD models. We tested immunomodulatory properties of *Faecalibacterium prausnitzii* strain in DSS model and we have found anti-inflammatory effects after *F. prausnitzii* application. The immunomodulatory effects were mediated through the TLR2-dependent modulation of IL-12 and IL-10 cytokines production in antigen presenting cells, suggesting that it contributes to the anti-inflammatory potency of *F. prausnitzii* strain HTF-F. The results showed that *F. prausnitzii* HTF-F and its extracellular polymeric matrix may have a therapeutic effect in IBD. (Rossi et al., PLoS One. 24:10, 2015).

● Gut microbiota plays important function in the biotransformation of xenobiotics. To investigate the effect of microbial colonization on messenger RNA expression of liver cytochromes P450, the main drug-metabolizing enzymes, we used gnotobionts: germ-free (GF) mice, mice monocolonized by non-pathogenic bacteria *Lactobacillus plantarum* ^{NIZO2877} or probiotic bacteria *E. coli* Nissle 1917 compared to specific pathogen-free (SPF) mice. We observed that colonization by non-pathogenic bacteria alters mRNA expression of cytochromes P450 in originally germ-free mice (Jourova et al., Folia Microbiol. 62:463, 2017). We analyzed the effect of microbiota on a non-steroidal anti-inflammatory drug nabumetone. At first we cultivated nabumetone with the selected gut commensal and probiotic bacteria under both aerobic and anaerobic conditions and analysed its metabolites by high-performance liquid chromatography. To analyze the effect of microbiota on nabumetone pharmacokinetics in vivo, we administered a single oral dose of nabumetone to rodents with intentionally altered gut microbiome. We found that nabumetone is metabolized by bacteria to its non-active

metabolites and that this effect is stronger under anaerobic conditions (Jourova et al., Xenobiotica. 49:1296, 2019).

● The purpose of our next project was to determine whether conventional or germ-free conditions and probiotics influence the response to psychosocial stress in brain and peripheral tissues and whether this effect depends on the postnatal development and programming of the stress axis. The project clarified mechanisms of action involved in the gut microbiota effects on brain neurochemistry, behavior and activation of the hypothalamus-pituitary-adrenal axis. We ascertained that the presence of microbiota modulates the stress response of the peripheral components of the hypothalamus-pituitary-adrenal axis (HPA) (pituitary and adrenal glands), the mesenteric lymphatic nodes and the colon. Microbiota modulated the efficiency of the negative feedback of the HPA axis at the level of pituitary gland and decreased the response of adrenal gland to stress. In contrast, microbiota upregulated the expression of cytokines in colon but stress strongly downregulated their expression. The project further showed that both chronic stress and microbiome modulate colonic peripheral metabolism of glucocorticoids and that these changes depend on cytokines. The effect of stress was not limited to the intestine, but we also demonstrated the modulatory effect of stress on peripheral glucocorticoid metabolism in lymphoid organs. (Ergang et al., Steroids 126:66, 2017; Vodička et al., Brain Behav Immun. 73:615, 2018). Gut microbiota is known to influence brain functions and behavior, however little is known about the effect of microbiota on stress response in distinct structures of HPA axis and intestine. In ongoing project we showed that microbiota impacts the response of pituitary, adrenals and intestine to stress and that interaction between stress and microbiota during activation of glucocorticoid biogenesis differs between adrenal gland and intestine (Vagnerova et al. Front Immunol. 10:2655, 2019).

● It seems that bacteria composition is among the possible cause of the development of diabetes in predisposed individuals. Therefore, we colonized germ-free non obese (NOD) mice with two bacteria representing groups considered modulators of diabetes penetration in predisposed individuals, namely *Bacteroides doreia* or *Akkermansia muciniphila*. Germ-free NOD mice were monocolonized at 3 - 4 weeks of the age and monoassociated NOD females were used to monitor the incidence of diabetes for 300 days. The insulitis and flow cytometric determination of subpopulations of regulatory CD4 + Foxp3 +, CD4 + CD45RBlow and CD4 + CD62L T cells, gamma / delta T cells, and inducible peripheral Tr1 (CD4 + CD49b + LAG3 + Foxp3-) T cells, and their cytokine profiles (IL-10, IFN-gamma) in mucosal (mesenteric and pancreatic) nodes were evaluated. At the end of the 10-month follow-up development in originally GF mice of the spontaneous incidence of diabetes has proven. (Neuman et al. Diabetologia 62:1291, 2019).

The Immune Regulations research group under the leadership of **Igor Splichal** targets to the innate immune response against enteric infections caused by *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium). *S*. Typhimurium naturally provokes self-limiting enterocolitis (salmonellosis) in humans and pigs, however, human typhoid fever-like illness in mice. In contrast to common patients, it can cause systemic illness in immunocompromised hosts as preterm infants. In our experiments, we used both the specificity of placentation of the pig and defined colonization of the gastrointestinal tract of gnotobiotic piglets to elaborate the gnotobiotic piglet model of preterm infants.

The first step of our effort was to modify conditions of breeding of highly immature piglets in sterile conditions and describe their peculiarity in selected histological and immunological parameters of the highly immature intestine compare to their term counterparts (Splichalova et al., 2018, Front Immunol, 9:220). This study involved histology of the immature intestine, tight junction proteins claudin-1 and occludin, pattern-recognizing receptors, adaptor molecules and co-receptors (TLR2, TLR4, TLR9, MD-2, CD14, MyD88, TRIF, and RAGE), and inflammasome NLRP3 transcription. On the protein level, we detected inflammatory cytokines interleukin (IL)-1□, IL-4, IL-6, IL-8, IL-10, IL-12/23 p40, tumor necrosis factor (TNF)-□, interferon (IFN)-□, and high mobility box group (HMGB) 1 protein in the ileum and colon of the preterm and term germ-free piglets. We found that in the preterm germ-free piglets, the ileum showed decreased lamina propria cellularity, reduced villous height, and thinner and less distinct stratification – especially muscle layer, in comparison with their term counterparts. Claudin-1 transcription increased in the intestine of the preterm piglets. The transcription levels of pattern-recognizing receptors and adaptor molecules showed ambiguous trends between the groups. The levels of IL-6, IL-8, IL-10, and TNF-□ were increased in the preterm ileum numerically (though not significantly), with statistically significant increases in the colon. Additionally, IL-12/23 p40 and IFN-□ were statistically significantly higher in the preterm colon. This work was supported by the Grant of the Czech Science Foundation No 13-14736S.

● We summarized our long-term experience with experimental enteric infections in the gnotobiotic piglet model in the Letter to the Editor in the J Inf Dis (Splichal and Splichalova, 2018, J Infect Dis, 218:504 – projects of the Czech Science Foundation).

● We used this preterm animal model in our following study of possible amelioration of *S*. Typhimurium-caused enterocolitis by previous colonization with probiotic *Lactobacillus rhamnosus* GG. The infection with *S*. Typhimurium caused clinical signs of enterocolitis, increased claudin-1, and IFN-□ transcriptions, but decreased occludin transcription, and increased local and systemic levels of IL-8 and IL-12/23 p40. Previous colonization with *L. rhamnosus* GG reduced *S*. Typhimurium colonization in the jejunum and translocation into the liver, spleen, and blood. It partially ameliorated histopathological changes in the intestine, reduced IL-8 levels in the jejunum and plasma, and IL-12/23 p40 in the jejunum (Splichalova et al., Clin Exp Immunol, 195:381, 2019).

● In collaboration with Prof. Sharon M. Donovan (Department of Food Science and Human Nutrition, University of Illinois, Urbana, IL, USA) and Prof. Eva Vlkova (Dept of Microbiology, Nutrition, and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Prague, Czech Republic) we studied impact of commensal and probiotic bacteria on *S*. Typhimurium-caused salmonellosis. These experiments were performed in the term gnotobiotic piglets. Mono-colonization of a piglet gastrointestinal tract with commensal *Lactobacillus amylovorus* and *Lactobacillus mucosae*, and with probiotic *E. coli* Nissle 1917 and their interference with *S*. Typhimurium infection were compared. We evaluated clinical signs, bacterial translocation, intestinal histology, mRNA expression of villin 1, claudin-1, claudin-2, and occludin in the ileum and colon, and local intestinal and systemic levels of inflammatory cytokines IL-8, TNF-□, and IL-10. Both lactobacilli colonized the gastrointestinal tract in approximately 100x lower density compared to *E. coli* Nissle and *S*. Typhimurium. Neither *L. amylovorus* nor *L. mucosae* suppressed the

inflammatory reaction caused by the 24 h infection with *S*. Typhimurium. In contrast to both lactobacilli, the probiotic *E. coli* Nissle 1917 was able to suppress clinical signs, histopathological changes, the transcriptions of the proteins, and the inductions of the inflammatory cytokines (Splichal et al., 2019, Microorganisms, 7 – grants of the Czech Science Foundation and the Grant Agency of the Charles University).

● In the following study with the same collaborators we paid our attention to the HMGB1 and TLR4 signaling (Splichal et al., 2019, Int J Mol Sci, 20). HMGB1 can be actively secreted by immune cells after different immune stimuli or passively released from cells undergoing necrosis. The released HMGB1 amplifies inflammation, and its hypersecretion contributes to multiple organ dysfunction syndrome and death. Transcription of HMGB1 and Toll-like receptors (TLR) 2, 4, and 9 and receptor for advanced glycation end products (RAGE), TLR4-related molecules (MD-2, CD14, and LBP), and adaptor proteins (MyD88 and TRIF) in the ileum and colon were evaluated. Expression of TLR4 and its related molecules were highly upregulated in the *S*. Typhimurium-infected intestine, which was suppressed by *E. coli* Nissle 1917, but not *L. amylovorus* nor *L. mucosae*. In contrast, HMGB1 expression was unaffected by *S*. Typhimurium infection or commensal/probiotic bacteria administration. HMGB1 protein levels in the intestine were increased in ST-infected piglets, but they were decreased by previous colonization with *E. coli* Nissle 1917 only. We concluded that the stability of HMGB1 mRNA expression in all piglet groups could show its importance for DNA transcription and physiological cell functions. The presence of HMGB1 protein in the intestinal lumen probably indicates cellular damage. Less effective lactobacilli grow in relatively low density. A prebiotic support of the growth of lactobacilli and multistrain lactobacilli inoculum could show higher protective effects. This work was supported by grants of the Czech Science Foundation and the Grant Agency of the Charles University).

● In another study, we evaluated the impact of lipopolysaccharide (LPS) chemotype on virulence of *S*. Typhimurium strain LT2 (Splichalova et al., 2019, Toxins (Basel), 11). One-week-old germ-free piglets were orally colonized/infected with the *S*. Typhimurium LT2 strain or its isogenic rough □*rfaL*, □*rfaG*, or □*rfaC* mutants with exactly defined lipopolysaccharide (LPS) defects. After 24 h wild-type *Salmonella* Typhimurium showed the highest translocation, histopathological changes, upregulation of claudins 1 and 2 and downregulation of occludin, transcription of the cytokines, intestinal IL-8, and TNF-□ levels, and systemic TNF-□ and IL-10 levels. Depending on the extent of the incompleteness of the LPS, the levels of the respective elements decreased, or no changes were observed at all in the piglets colonized/infected with □*rfa* mutants. Intestinal IL-10 and systemic IL-8 levels were not detected in any piglet groups. This study provided foundational data on the gnotobiotic piglet response to colonization/infection with the exactly defined rough S. Typhimurium LT2 isogenic mutants. The work was supported by a grant of the Ministry of Education, Youth and Sports. This work was done also in collaboration with Dr. Paolo Trevisi, University of Bologna, Italy in the frame of EU project COST.

The research group of **Tomas Hrncir** is primarily focused on the effects of food xenobiotics on the composition and function of human gut microbiota, the immune system modulation, and the consequences of these alterations for human health. In recent years, the group has focused mainly on the impact of common food preservatives. Specifically, we have tested the hypothesis that that antimicrobial food

additives may alter the composition of human gut microbiota by selectively suppressing the growth of susceptible gut microbes. To explore the influence of antimicrobial food additives on the composition of the human gut microbiota, we examined the susceptibility of both aerobic and anaerobic gut bacteria to sodium benzoate, sodium nitrite, and potassium sorbate, and their combinations, using a broth microdilution method. The tested bacteria exhibited a wide range of susceptibilities to food additives. For example, the most susceptible strain, *Bacteroides coprocola*, was almost 580 times more susceptible to sodium nitrite than the most resistant strain, *Enterococcus faecalis*. However, most importantly, we found that gut microbes with known anti-inflammatory properties, such as *Clostridium tyrobutyricum* or *Lactobacillus paracasei*, were significantly more susceptible to additives than microbes with known proinflammatory or colitogenic properties, such as *Bacteroides thetaiotaomicron* or *Enterococcus faecalis*. Our data show that some human gut microbes are highly susceptible to antimicrobial food additives. We speculate that permanent exposure of human gut microbiota to even low levels of additives may modify the composition and function of gut microbiota and thus influence the host's immune system. Whether the effect of additive-modified gut microbiota on the human immune system could explain, at least in part, the increasing incidence of allergies and autoimmune diseases remains to be shown. The above-mentioned findings from the *in vitro* experiments were published recently (Hrncirova et al., Folia Microbiol 64:497, 2019).

- To extend our *in vitro* work and to confirm or reject the hypothesis that food preservatives may induce gut microbiota alterations, i.e., dysbiosis, associated with many immune-mediated and metabolic diseases, we have generated human microbiota-associated mice. Using this model on wild-type and Nod2-deficient background, we have shown that a mixture of common antimicrobial food additives can induce dysbiosis characterized by an overgrowth of *Proteobacteria* phylum and a decrease in the *Clostridiales* order. Remarkably, human gut microbiota in a *Nod2*-deficient genetic background was even more susceptible to the induction of *Proteobacteria* dysbiosis by additives than the microbiota in a wild-type background. Our data convincingly prove that antimicrobial food additives are capable of triggering gut microbiota dysbiosis in both wild-type and *Nod2*-deficient backgrounds and at the exposure levels reached in European populations. Whether this additive-modified gut microbiota plays a significant role in the pathogenesis of immune-mediated and metabolic diseases remains to be elucidated. These data were published recently (Hrncirova et al., Microorganisms 7, 2019).

- The group is also interested in the research of pathogenetic mechanisms of autoimmune uveitis and the potential role of gut microbiota in its induction. We believe that a better understanding of the stimulatory effects of gut microbiota during the activation of autoreactive immune cells might enable novel therapeutic approaches exploiting the better knowledge of microbiota-immune system interactions. So far, we have found that germ-free mice and the mice with antibiotic-depleted gut microbiota are protected against the development of severe autoimmune uveoretinitis. These mice have lower numbers of infiltrating macrophages and T cells in the retina compared to conventional mice. Also, these mice had reduced numbers of IFN-g and IL-17-producing T cells and increased numbers of regulatory T cells in eye-draining lymph nodes. These data suggesting that the presence of microbiota during autoantigen recognition regulates the inflammatory response by influencing the

adaptive response were published in *the Journal of Immunological Research* (Heissigerova et al., J Immunol Res. 5065703, 2016.) However, this research is far from finished and is still ongoing.

● Another research area, in cooperation with Tomas Hudcovic, is defining the role of gut microbiota in modulating emotional and stress responses. It is known that the commensal microbiota affects brain functioning, emotional behavior, and ACTH and corticosterone responses to acute stress. However, little is known about the role of the microbiota in shaping the chronic stress response in the peripheral components of the hypothalamus-pituitary-adrenocortical (HPA) axis and in the colon. In recent years, we have studied the effects of the chronic stress-microbiota interaction on HPA axis activity and on the expression of colonic corticotropin-releasing hormone (CRH) system, cytokines, and 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), an enzyme that determines locally produced glucocorticoids. Using specific pathogen-free (SPF) and germ-free (GF) BALB/c mice, we have shown that the microbiota modulates emotional behavior in social conflicts and the response of the HPA axis, colon and mesenteric lymph nodes (MLN) to chronic psychosocial stress. In the pituitary gland, microbiota attenuates the expression of Fkbp5, a gene regulating glucocorticoid receptor sensitivity, while in the adrenal gland, it attenuates the expression of genes encoding steroidogenesis (MC2R, StaR, Cyp11a1) and catecholamine synthesis (TH, PNMT). However, we have found that the pituitary expression of CRH receptor type 1 (CRHR1) and proopiomelanocortin is not influenced by microbiota. In the colon, the microbiota attenuates the expression of 11HSD1, CRH, urocortin UCN2, and its receptor, CRHR2, but potentiates the expression of cytokines TNFα, IFNγ, IL-4, IL-5, IL-6, IL-10, IL-13, and IL-17, with the exception of IL-1β. Compared to GF mice, chronic stress upregulates the expression of pituitary Fkbp5 and colonic CRH and UCN2 in SPF mice and downregulates the expression of colonic cytokines. Differences in the stress responses of both GF and SPF mice were also observed when the immunophenotype of mesenteric lymph node cells and their secretion of cytokines were analyzed. Our data suggest that the presence of microbiota/intestinal commensals plays an important role in shaping the response of peripheral tissues to stress and indicates possible pathways by which the environment can interact with glucocorticoid signaling. Our work on stress responses and gut microbiota was published in *Brain Behaviour and Immunity* (Vodička et al., Brain Behav Immun 73:615, 2018).

Research activity and characterisation of the main scientific results

Tumour microenvironment (TEM) immunology is the main research topic with focus on immunity and tissue remodelling relationships.
During the period 2015-2019, the laboratory of immunotherapy was following its investigation about the tumour microenvironment immunology according to the lines regarding: 1) local immunity and tissue structure modifications; 2) nanoparticles for theranostic and tumour targeting; 3) natural products for immunomodulation and new experimental models. We can summarise the main results as following:

1) Immunity and tissue structure modifications:

1a) Rat model

We completed the analysis of the data accumulated in previous studies about dextran sodium sulphate (DSS) colitis induction and azoxymethane (AOM) carcinogenesis induction in rats. This was the project followed by a PhD student, Fabian Caja. The final evaluation of the data of cytokine expression (ELISA, qRT-PCR) and collagen density modification (by 2-photon confocal analysis in second harmonic generation mode, in collaboration with the Laboratory of Biomathematics, Institute of Physiology of the Czech Academy of Sciences, Prague) at 1 month after induction showed significant differences in the collagen scaffold between each evaluated group (control, DSS, AOM) (Fig.1) together with an unexpected general downregulation of inflammatory and regulatory cytokines. These results showed a re-modulation of the inflammatory signals with their possible maintenance associated to reduction of regulatory signals. However, even reduced, the pro-inflammatory signals (IL-1α/β, IL-6) in the carcinogenesis model still appear relevant in relation to the downregulated regulatory signals (TGFβ). This is suggesting the idea of variable threshold of inflammation. These data are already collected in a paper, currently under language review before sending to publication.
The importance of the collagen scaffold, inflammation and of TGFβ were discussed in the following papers [1,3,6] and one that is under publication [16]. This paper is part of the doctoral study of a PhD student, Paolo Tenti, with topic on LOX molecules expression in the inflammatory and cancer induced tissues in the early stages of the tumour microenvironment organisation.

1b) mouse model - evaluation of structural changes induced in the colon mucosa after conventionalization of GF mice

In consideration of the results previously obtained about the structural changes, induced by inflammation in the colon of rats under CV and GF conditions, we evaluated the changes in the colon mucosa induced by conventionalization of GF mice introduced in regular rearing conditions. The results showed a quick remodelling of the mucosal scaffold, reaching in 1 week the complexity as in CV animals (Fig.2). This was associated to the modification of cytokine levels and their reciprocal relationship; not only at local level but also at thymus level (Fig.2). In the thymus, this correlate also with the observation of temporary migration of special type of thymic macrophages (metalophylic macrophages) from medullar to cortical sites (Fig.2). This type of macrophages is considered related to the antigen presentation and possibly to tolerance mechanisms. These results are under compilation for a paper that will be in collaboration with the University of Belgrade, where the macrophage study was performed.

This study confirmed the very active influence of immune signals on quick remodelling of a tissue scaffold, as observed in previous experiment in the rat model. Moreover, in the previous experiment was found that DSS induced inflammation produce heavy alterations of the collagen scaffold in the CV animals (with already activated immunity in the colon), while induce maturation in the scaffold in GF animals (which miss continuous stimulation by the commensal microbiota). Taken all together, our reported observations strongly suggest that inflammatory signals are fundamental for the tissue organization: the changes are extremely quick and depending on the balance of pro-inflammatory and regulatory signals. Therefore, we can also propose a hypothesis of existence of an inflammatory threshold sustaining the tissue homeostasis in each tissue and its level of response to pathological or modified stimuli. Regular stimuli may become pathological if the threshold is changing. These results were time by time partially presented in various international conferences and are progressively prepared for publication.

1c) **A comparative study on human carcinoma specimen** was performed in collaboration with the Department of Surgery, 1st Faculty of Medicine, Charles University, Thomayer Hospital in Prague. We analysed in qRT-PCR, the gene expression of pro-inflammatory and regulatory cytokines from samples of normal mucosa far from the tumour, mucosa close to the tumour and tumour tissue. We found an increase of structural molecules (COL1A1, LOXL2) as well as pro-inflammatory cytokines (IL-1β, IL-6, IL13, IL-17) gene expression while downregulation of the regulatory signals (IL-10, TGFβ1) in the transitional zone between normal mucosa and tumour. Interestingly, all this was associated to the increase of immune checkpoint protein gene expression (PD-1, PD-L1) in the same tissue (Fig.3). These results can suggest important crosstalk between local immunity and tissue scaffold remodelling able to even influence the local levels of immune response by regulating the expression of immune checkpoint proteins. New samples are under analysis to complete the data and confirm the present hypothesis.

2) Nanoparticles for theranostic and tumour targeting

Within the various possible approaches to modulation of the tumour microenvironment, we study the possibility to use nanocarriers (nanoparticles = NP) giving the possibility to be both diagnostic and therapeutic. This theranostic approach can be obtained by the specific characteristics of NPs. They can bring molecules able to be detected (Gadolinium) and/or loaded with therapeutic drugs and theoretically, they can reach and accumulate even in complex environments. About the possibilities of heard NPs see the chapter [11].

Following the study of human ferritin-based NPs, performed in collaboration with Institute of Molecular Biology and Pathology - CNR and University La Sapienza, Rome, Italy, as published in [2], we evaluated Gadolinium-based NPs (Gd NPs) (in collaboration with Institute of Macromolecular Chemistry, Prague, prepared by Ing. Kostiv), Ferric-based NPs (Fe NPs) (in collaboration with Institute of Macromolecular Chemistry, Prague, prepared by Dr. Babic) and TiO2 nanospheroids (in collaboration with the Vinca Institute of Nuclear Sciences, Department of Atomic Physics, Belgrade, Serbia). All studies were performed to evaluate the possible cytotoxicity and immune effects of NPs in the view of their utilisation as a theranostic materials.

2a) The study about Gd NPs was developed in the frame of the COST TD1301 MiMed (Dr. Vannucci – Czech manager and leader of the theranostic group) and COST BM1309 EMF-MED. In the frame of COST action TD1301 MiMed was developed the grant COST CZ LD 15135 by the Czech Ministry of Education, including the study of Gd NPs. The results of the Gd study are published in a paper

[5] and, in summary, demonstrated that the level of toxicity of Gd NPs was very limited even in comparison with the regular Gd for magnetic resonance (MR) with low immunotoxicity and capability to be very well traced at the MR evaluation (in collaboration with Institute for Clinical and Experimental Medicine, Prague). Analysis by laser ablation inductively coupled plasma mass spectrometry LA-ICP-MS (in collaboration with Laboratory of Molecular Structure Characterization, Institute of Physiology of the Czech Academy of Sciences, Prague) demonstrated localization of the Gd NPs at the level of vascular wall in the tumour and organs (brain).

2b) In collaboration with the Vinca Institute of Nuclear Sciences, Department of Atomic Physics, Belgrade, Serbia, we tested TiO2 nanospheroids suitable for photo-activation and cytotoxic photodynamic effect on tumour cells. In our contribution to this collaborative study with the institute, we had part in the evaluation of effects on splenocytes by MTT at different time schedules (0h, 24h, 48h).

Immediate reduction of viability of the splenocytes after the treatment was followed by quick recovery at the following tested time points. The full results of the study will be soon available in the paper [17] (under review).

2c) A wider study is ongoing on Ferric superparamagnetic NPs in collaboration with Institute of Macromolecular Chemistry, Prague. Superparamagnetic NPs are of interest, because they can be used for heating by Joule effect when inserted in adequate electromagnetic field. By this way, they could be suitable for both imaging and *in situ* activation for tumour microenvironment treatments. 4 types of NPs are currently under study: Fe2O3 NP naked and coated with dimethylacrylamide-co-acrylic acid (DMA-AA) and complexed with nickel to increase their signal in MR (in collaboration with Institute for Clinical and Experimental Medicine, Prague) and for detection by LA-ICP-MS (in collaboration with Laboratory of Molecular Structure Characterization, Institute of Physiology of the Czech Academy of Sciences, Prague). Before this kind of test, their possible cytotoxicity and immunotoxicity was evaluated. As a first step were performed tests by MTT and Crystal violet for viability and apoptosis and ROS evaluation by FACS analysis. The study is performed on mouse cell lines; non-tumoural (3T3) and tumoural (CT26). Present results indicate that only the naked Fe NP complexed with nickel show slightly reduced viability that is dose dependent and in agreement with observed increased apoptotic rate after 48h incubation (Fig.4). This effect was more profound in the tumour cell line CT26. None of the NPs seemed to have effect on oxidative state of both cell lines. Subsequently, all NPs were systemically administered *in vivo* to Balb/c mice and 24h after administration tested for immunotoxicity by FACS analysis. No significant changes were observed. The only exception was a slightly reduced percentage of NK/NKT cells and mild trend in activation of B lymphocytes in the spleen (Fig.5).

The study, that is a PhD topic of Lenka Rajsiglova, is still continuing. Part was performed by the student in 2018 in the laboratory of prof. Adriana Albini at IRCSS Multimedica, Milan, Italy.

3) Natural products for immunomodulation and new experimental models

3a) Another approach to the immunomodulation of the tumour microenvironment is by the utilisation of molecules derived from natural sources. Thanking a collaboration with University of Louisville, Department of Pathology, KY, USA, in this year, we were reviewing the activity of β-glucans in human and veterinary applications. The widely performed review of literature indicated the value of β-glucans as positive modulators of immunity and their utility even in metabolic diseases (e.g. hypercholesterolemia, diabetes) [7,8,9,13,14,15].

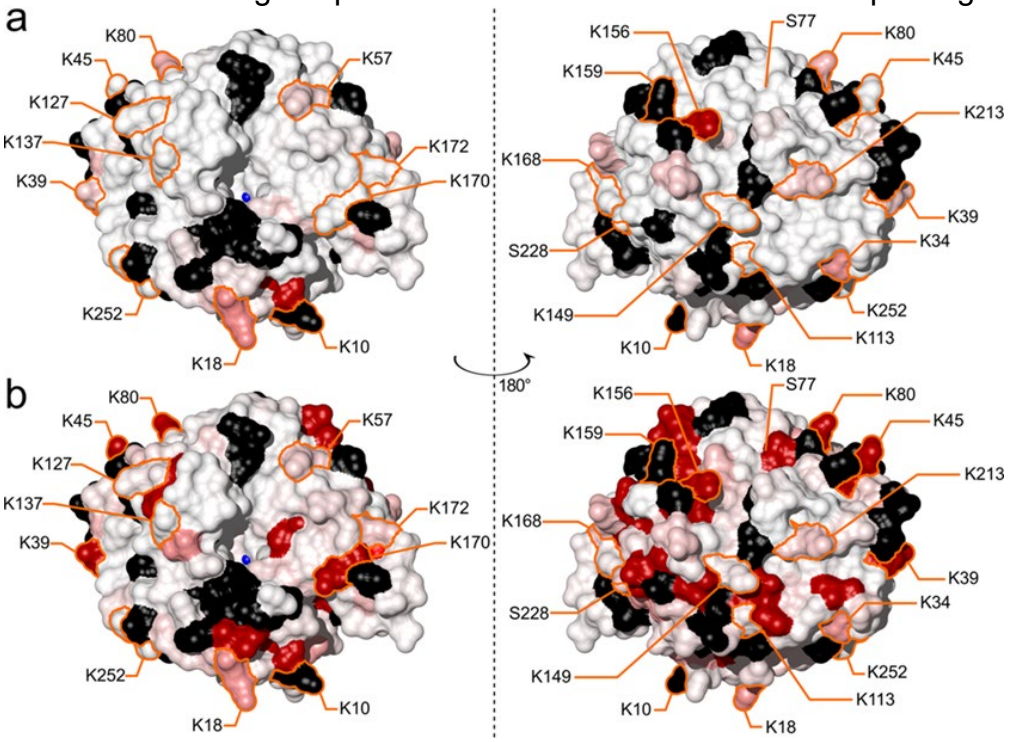
Regarding the protective roles of β-glucans and their use in humans and animals and their possible application as a treatment, even in cancer, it resulted clear the necessity to activate studies for a more precise definition of the kind and role of polysaccharides derived from various sources and the specific effects of each obtained compound.
The evaluation of newly extracted polysaccharides (CPS1, CPS2, CPS3) from heterotrophic mutant of green microalgae *Chlorella vulgaris* G11 was performed thanking the collaboration with the department of Carbohydrates and Cereals of the University of Chemistry and Technology in Prague. First part of the evaluation was done by ELISA, MTT, ImmunoSpot and FACS analysis. The preliminary results showed that CPS2 polysaccharide was responsible for an increase in the production of INF-y in the melanoma group, compared with the control group and the other cytokines. We didn't find changes in the remaining cytokines (TNF-α, IL-4) between tumour and non-tumour bearing animals. The result shows possible immunomodulatory changes toward Th1 response.
A paper is under review [18]; the study is continuing. First study was commonly conducted by PhD students Pavol Lukac, Lenka Rajsiglova and Leonid Sushytskyi from University of Chemistry and Technology, Prague.
3b) In the study of microenvironment is involved use of *in vivo* models. However, to reduce the number of experimental animals to be used and to obtain standardise models that can help quicker evaluation of mechanisms and responses, for example, to therapeutic procedures, we started to establish 3D cultures of non-tumoural and tumoural cell lines. Spheroids of 3T3, CT26 and B16F10 murine cell lines and HT29 human cell line were performed for evaluation of growth rate *in vitro* and general assessment of the spheroids behaviour. The spheroid morphology and assessment were evaluated by direct staining of cell membranes and nuclei and examined in confocal microscopy, measured and reconstructed in 3D imaging. A first examination of interesting markers expressed by tumour in its development, was done using antibodies against PD-L1 immune checkpoint molecule. CT26 colorectal carcinoma mouse cell line is expressing PD-L1 and was used for this initial test. The comparison between the *in vitro* expression of PD-L1 in 2D culture vs. 3D culture suggest some possible difference in expression between cells that are on surface of the spheroid and cells that are in more inner layers (Fig.6). This study is initial and will be continued by a PhD student Pavol Lukac as a topic of his PhD study, looking to the development of organoids and complex spheroids with more cell types to resemble the tumour microenvironment.

Research activity and characterization of the main scientific results

Since the LSBCS represents a multidisciplinary research team covering a broad area of interest from cutting edge mass spectrometry through clinical diagnostics towards cell signaling the research activities of the laboratory can be divided into the three research topics:

1. Development of stable covalent labeling methods for protein-protein and protein-nucleic acid interaction structural characterization.
2. Methods and software development for hydrogen-deuterium exchange
3. Design of protein affinity chips for clinical diagnostics

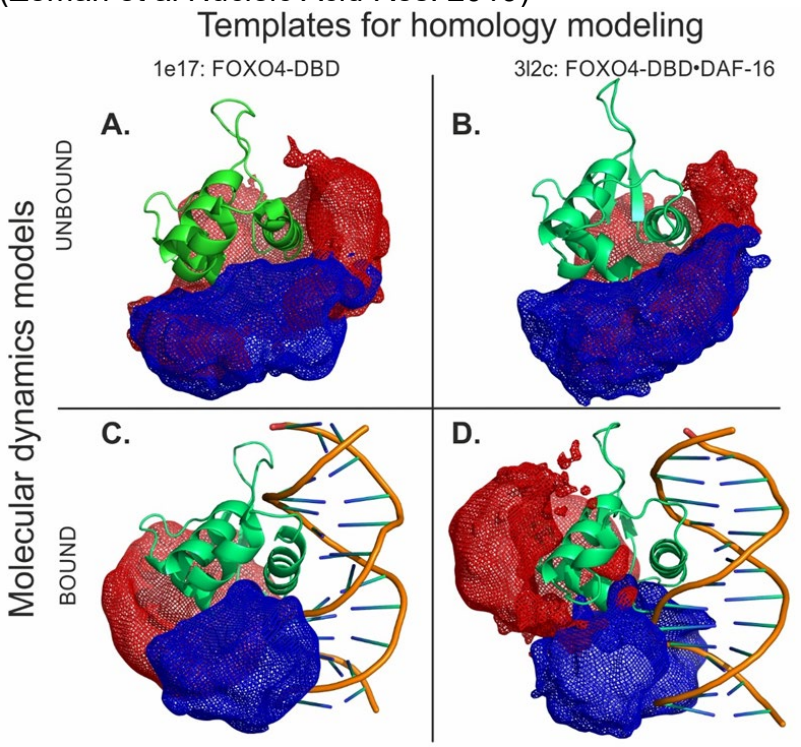
The first topic represents the development of cross-linking technique for structural characterization of proteins alone or as a part of biomolecular complexes including dsDNA and testing the potential of novel stable covalent foot printing methods.



Structural changes of hCA-I mapped onto the crystal structure upon cross-linking with 10 molar excess (a) and 100 molar (b) excess of BS2G. Residues are colored in the red gradient to show the intensity of chemical shift change. The residues that could not be unambiguously assigned due to overlap or significant decrease of the intensity are colored black. The zinc atom in the active site is colored blue.

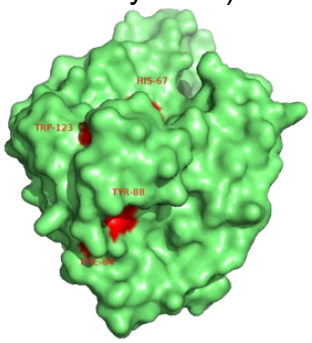
Even our team has proven a long term history in chemical cross-linking and stable covalent labeling we focused on optimization of cross-linking reactions believing the yield of the reaction should not be increased using higher amount of chemical probe but doing fine tuning of cross-linking protocol. To prove our idea, the model protein human carbonic anhydrase was cross-linked using different molar ration of chemical probes over the protein and various protein concentrations. The results of cross-linking reactions were analyzed by mass spectrometry, nuclear magnetic resonance and

measuring enzyme kinetics. Our data clearly demonstrated that the increasing concentration of protein or chemical probes induced conformational rearrangement (see picture above) within protein structure and suggest optimal ration of protein vs. chemical probe for cross-linking reaction (Rozbesky et. al. Anal. Chem. 2018). The optimized protocol was subsequently used not only for internal experiments (e.g. Hernychova et al J. Prot. 2019). It was also adopted to proceed samples of our collaborators (e.g. Hnizda A. et al BMC Biol. 2016, Hnizda A. et al Leukemia 2018 and Sharma S. et al Proc. Natl Acad Sci USA 2018) and it was also used for the international comparative cross-linking study (Iacobucci C. at al Anal. Chem. 2019). In parallel, we investigated the potential of isotopically labeled chemical probes to map the conformational changes of proteins induced by ligand binding. The holo form of Calmodulin (Ca^{2+} containing protein) was modified with the light chemical probe and the apo form (without calcium) with the heavy cross-linker. Both reactions were quenched and mixed together. Structural differences between the holo and apo forms of Calmodulin were observed and they correlated with the known structural models of Calmodulin both forms very well (Kukacka et al Methods 2015). The quantitative cross-linking approach was further utilized to visualize structural changes of transcription factor DNA binding domain upon dsDNA binding (Slavata et al Biomolecules 2019). The last mentioned manuscript it is a result of collaboration with Prof. Fabris group and it doesn't report the utilization of quantitative cross-linking for protein-dsDNA structural study only. It also showed the complete mass spectrometry package to design structural model of protein-nucleic acid complexes. The hydrogen-deuterium exchange, quantitative protein-protein cross-linking, protein-nucleic acid cross-linking, homology modeling and molecular dynamics were utilized to design ab initio structural model of FOXO4 transcription factor and DAF16 (FOXO4 DRE sequence) complex (see the picture below). Suh technique was in part utilized for our collaborators as well (Zeman et al Nucleic Acid Res. 2019)



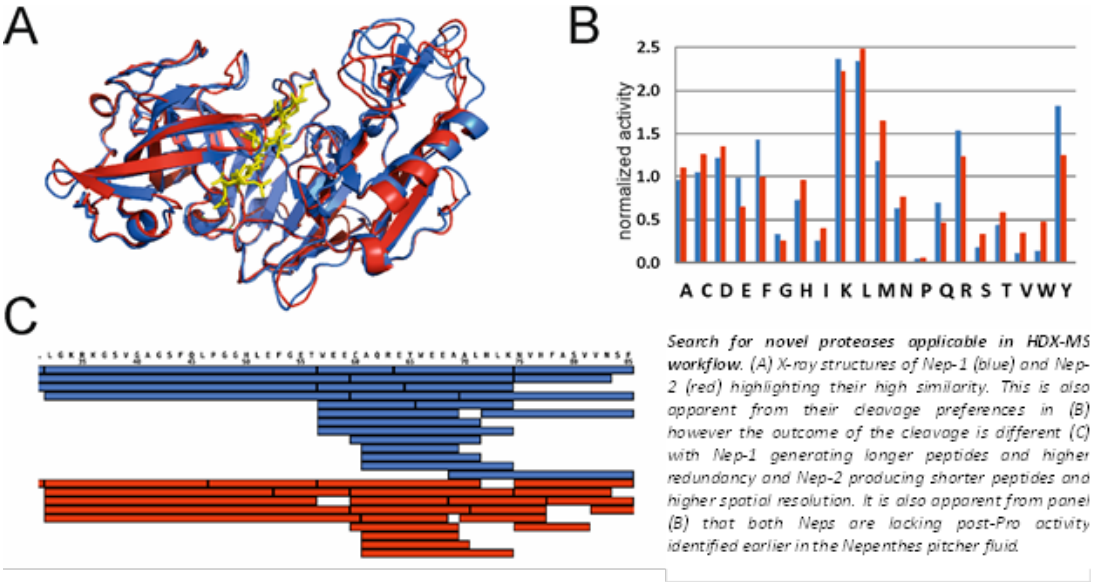
Structural proteomics could effectively guide model-building operations to produce very high-quality 3D models. Models of FOXO4-DBD and FOXO4-DBD•DBE were obtained by combining homology modelling with experimental

constraints and molecular dynamics simulations. These models incorporated extensive information from protein-DNA cross-links, quantitative protein-protein cross-links, and hydrogen-deuterium exchange. The green structures show representative models for unbound (A. and B.) and bound (C. and D.) forms based on corresponding 1E17 (A. and C.) or 3L2C (B. and D.) high-resolution templates. The mesh areas in blue and red colors represent the spaces occupied by all the models in the ensembles, which provided a measure of the flexibility of the N- and C- terminal regions.
Further, complementary to HDX (labeling of the polypeptide backbone) our lab started testing the potential of hydroxyl radical (labeling amino acid sidechains) foot printing for studying dynamics of proteins and protein-protein / protein-nucleic acid complexes in solution. We successfully adopted the fast photo-oxidation of proteins (FPOP) methods relaying on dissociation of hydrogen peroxide by KrF excimer laser enabling sub seconds labeling pulses and we are the pioneering lab offering such an expertise in Europe nowadays. In parallel to hydroxyl radical foot printing, we made a close collaboration with organic chemists from private company CF Chemical plus and Institute of Organic Chemistry and Biochemistry to define the potential of Togni reagents for protein structural studies. The Lewis acid activation of Togni reagents resulted in generation of Fluor alkyl radicals modifying amino acid sidechains of aromatic amino acids within the protein structure (picture below). The Ubiquitin and human carbonic anhydrase served as protein model systems (Rahimidashaghoul K et al Chemistry 2019).



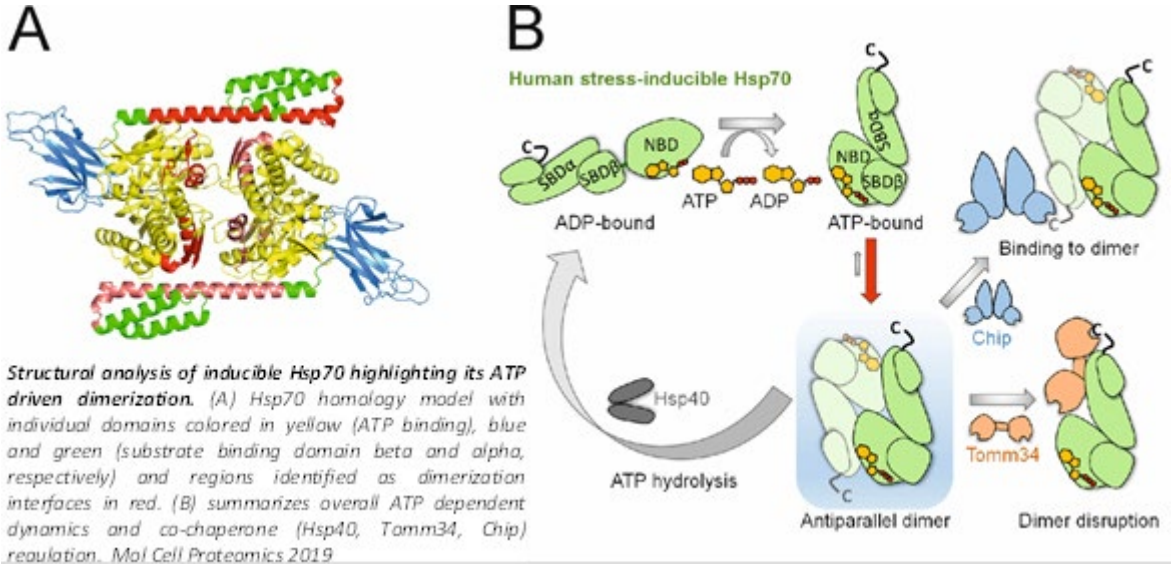
***Structural model (1hcb) of hCA I** with Trp123, Tyr88, Phe84 and His67 residues, which underwent fluoroalkylation highlighted in red.*

In the second activity, we dedicated significant amount of time and effort to the area of hydrogen/deuterium exchange mass spectrometry. Here both methodology development and applications were actively pursued. Spatial resolution in HDX-MS is reached via sample proteolysis. This step requires acidic proteases as it must be done rapidly, at low temperature and at low pH (2.5). We previously performed systematic screening of protease content in the pitcher of carnivorous plants genus Nepenthes and described expression and use of Nepenthesin-1. In further systematic search for additional pitcher fluid protease having unique specificity for proline residues, we did similar study for Nepenthesin-2 (Anal Chem 2015) in collaboration with David C. Schriemer from University of Calgary. Nep-2 was lacking the post-Pro cleavage but still provided considerable addition to protease toolbox in HDX-MS workflow as it again complemented widely used pepsin but interestingly also differed in cleavage pattern, stability and inhibition properties from previously described Nep-1. We used these proteases in different HDX-MS studies where they helped to access proteins not easily amenable by pepsin or improved spatial resolution of our HDX-MS studies (e.g BBA 2017, AnalChem 2019, Biomolecules 2019+2019). In addition, we performed crystallization and X-ray diffraction of both Nepenthesins in collaboration with Jan Dohnalek group from IBT (Acta Crystallogr F 2016). High-resolution structures are solved (see below) but not released yet as we work on their further



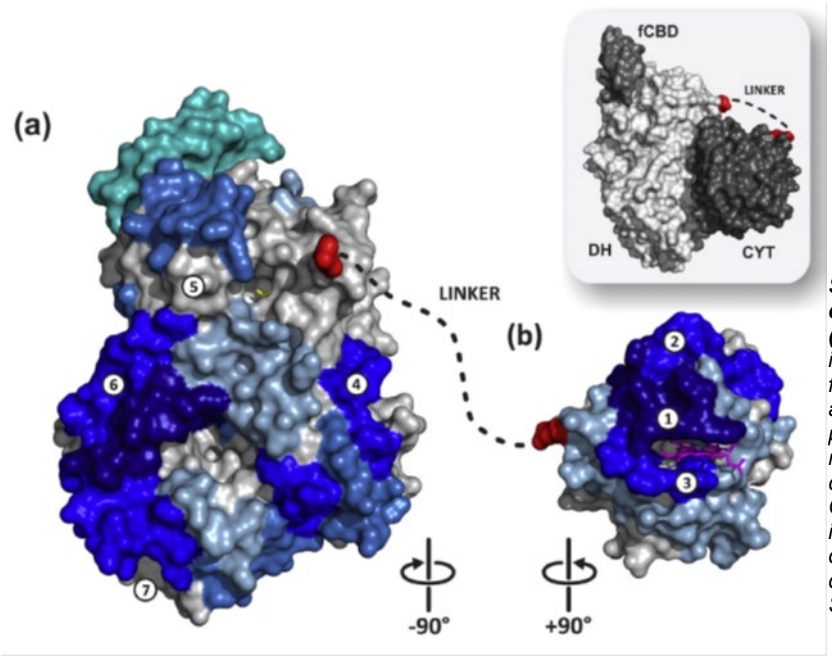
Search for novel proteases applicable in HDX-MS workflow. (A) X-ray structures of Nep-1 (blue) and Nep-2 (red) highlighting their high similarity. This is also apparent from their cleavage preferences in (B) however the outcome of the cleavage is different (C) with Nep-1 generating longer peptides and higher redundancy and Nep-2 producing shorter peptides and higher spatial resolution. It is also apparent from panel (B) that both Neps are lacking post-Pro activity identified earlier in the Nepenthes pitcher fluid.

improvement. We showed that while both enzymes are highly similar, they significantly differ in their stability, inhibition properties and also provide different cleavage products in HDX-MS proteolysis and thus we work further on explanation of these differences. It should be noted that the structure of Neps is the first one solved in this enzyme class. Comparison of previous results obtained on Nepenthes pitcher fluid with the specificities of recombinant Nepenthesins showed that the unusual post-proline cleavage must be exerted by another protease. Fractionation and activity screening lead to identification of 29kDa protease with highly selective post-Pro and post-Ala cleavage which was later cloned and expressed in collaboration with group of D.C. Schriemer. This novel protease was named Neprosin and represents completely new enzyme. Its cleavage pattern makes not only good addition to HDX-MS protocol but was shown to be, in combination with Nepenthesins, highly potent in digestion of gluten proteins. Since these are known as triggering agents of Celiac disease, Nepenthesins and Neprosin were further studied also in this context. This work was again collaborative approach done together with D.C. Schriemer's group and it resulted into two publications (Sci Rep 2016 and Mol Cell Proteomics 2017) and a US patent "TREATMENT OF GLUTEN INTOLERANCE AND RELATED CONDITIONS" which is now under evaluation for licensing by a company in US. Use of protease columns was then further extend by our lab also to studies dealing with protein modifications, as the non-specific digestion provides full sequence coverage and thus allow for complete characterization of protein primary sequence and localization of protein modifications (e.g. J Biol Chem 2016, FEBS J 2016, J Agric Food Chem. 2016, FEBS Open Bio 2018, BBRC 2017 or Biophys J. 2017). Beside the acidic proteases we also further developed our software for HDX-MS data processing named DeutEx (http://www.deutex.org/doc/doku.php + http://peterslab.org/downloads/SW/DeutEx.mp4) and extended previously published visualization tools on web-page MSTools - http://peterslab.org/MSTools/index.php.



Structural analysis of inducible Hsp70 highlighting its ATP driven dimerization. *(A) Hsp70 homology model with individual domains colored in yellow (ATP binding), blue and green (substrate binding domain beta and alpha, respectively) and regions identified as dimerization interfaces in red. (B) summarizes overall ATP dependent dynamics and co-chaperone (Hsp40, Tomm34, Chip) regulation. Mol Cell Proteomics 2019*

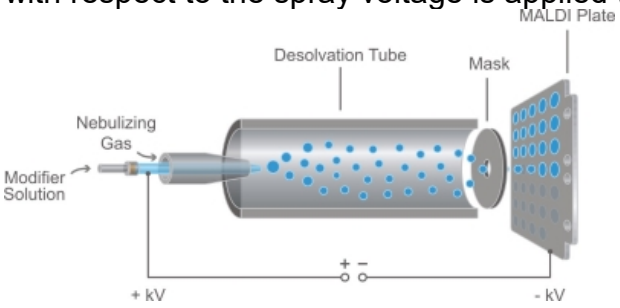
We then took advantage of all these improvements in obtaining structural insight into projects pursued in the lab (heat shock proteins, protein-DNA interaction, and cellulose degrading enzymes – FEBS Lett 2015, BBA 2017, Mol Cell Proteomics 2019, Biomolecules 2019+2019) or done in collaboration with other groups (J Biol Chem 2015, J Immunol 2015, Sci Rep 2016+2016, Proteins 2016, J Biol Chem 2017+2017, eLife 2017, Nat Commun 2017, Mol Cell Proteomics 2017, BBA 2019). Impact of our work also resulted into and invitation to participate in setting the guidelines in the field – Nat Methods 2019.



Structural changes in cellobiose dehydrogenase at different pH values (7.4 vs 5.4). *Blue tones highlight regions influenced by pH change showing that the flavin and cytochrome domain interfaces are becoming protonated at slightly acidic pH which removes the negative charge repulsion and allows for close interdomain contact and efficient electron transfer. Closed CDH conformation is shown in the inset in top right corner. The colored domains in (a) and (b) are rotated by 90 degrees for the sake of clarity. BBA Gen Subjects 2017*

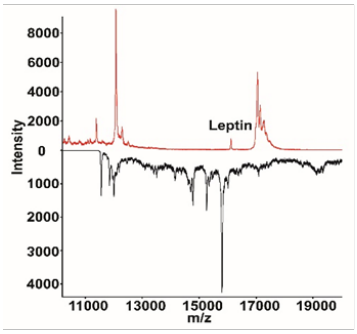
In the last pillar, the LSBCS successfully developed several applications based on the functionalized surfaces combined with MALDI mass spectrometry. The MALDI chips are design to be applied predominantly in life sciences and clinical diagnostics. The concept of ambient ion landing for preparation of functionalized surfaces was firstly developed several years ago in Graham Cooks laboratory and further modified by other groups. In the Laboratory of structural biology and cell signaling, functionalized

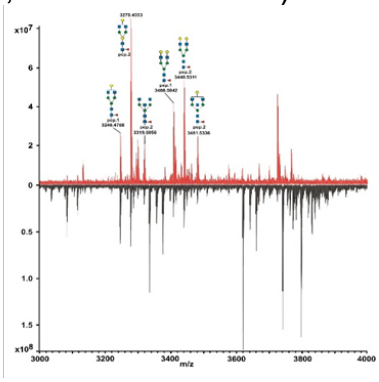
surfaced were firstly prepared by ambient ion landing under atmospheric pressure. This technology, which was developed at Institute of Microbiology, v.v.i. and is currently under patent protection, was successfully applied for in-situ enrichment and detection of phosphorylated peptides by MALDI mass spectrometry (Krasny et al.). The lab-made apparatus for modification of surfaces by proteins is composed of nanoelectrospray emitter on which a high voltage is applied, grounded desolvation tube and vertically mounted MALDI plate on which high voltage with opposite polarity with respect to the spray voltage is applied (see scheme).



***Scheme of the apparatus**. The heated tube is kept on ground, while the slit mask electrode is on high negative potential. The motion of the target is automated for array deposition.*

The results described in paper by Volny et. al. clearly show trypsin activity after its deposition on surface in vacuum. Our data show that functionalized MALDI plates with enzymes, lectin and antibody, prepared by the modified apparatus working at atmospheric pressure, are also suitable for on-plate digestion of proteins and for enrichment of glycopeptides and antigens from complex matrices. In all experiments, the landed protein always maintained its biological activity and was able to impose on the surface upon immobilization. The technology has the potential to be used for immobilization of any protein molecules that can survive electrospray ionization and ambient ion landing without a significant loss of activity. The success of the technique for preparation of MALDI compatible protein chips is based on the fact that ambient ion landing allows immobilization directly on a dry metal or metal oxide surface. The absence of any interlayer between conductive MALDI surface modified by ion landing and antibody affinity molecules reduces the non-specific interactions of other proteins in the sample and maintains the original conductivity of the MALDI plate, which provides efficient ionization. Figure below represents MALDI MS spectra of in-situ enriched leptin using surface modified by anti-leptin antibody and in-situ enriched glycopeptides from human IgG1 using surface modified with lectin wheat germ agglutinin (Pompach P. et al., Anal. Chem. 2016).

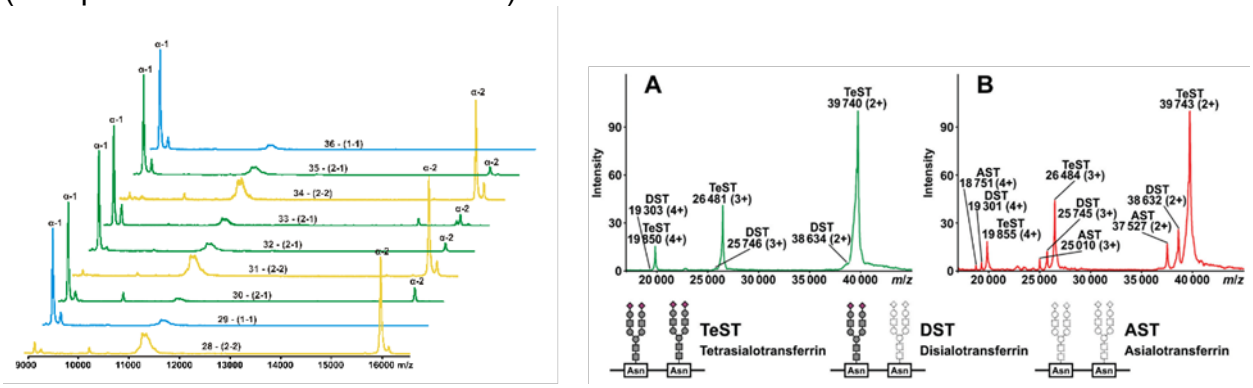




***Left-MS** spectrum of enriched (red) leptin and nonenriched sample (black). **Right-MS** spectrum of IgG1 glycopeptides bearing complex fucosylated glycans using MALDI chip functionalized with WGA lectin. The flipped black spectrum was obtained from the same sample without the in-situ enrichment.*

The successful enrichment and detection of leptin by functionalized surfaces led to idea to design assays for detection and quantification of other serum antigens e.g. haptoglobin phenotype. Haptoglobin has many clinical aspects and the knowledge of haptoglobin phenotype help for better prediction of several diseases progression including diabetes, hematologic disorders and cardiovascular disorders. In this project,

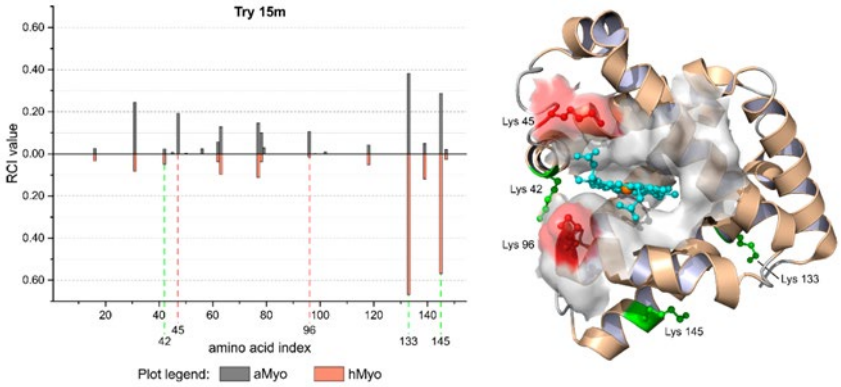
surfaces modified by ion landing procedure with anti-haptoglobin antibody were utilized for determination of human haptoglobin phenotype. The results of more than 100 cohorts revealed 100% accordance when validated by standard SDS electrophoresis followed by Western blot with immunodetection. Figure below represents detection of alpha subunits determining haptoglobin phenotype (Hp1-1, Hp2-1 and Hp2-2). The method for phenotyping of haptoglobin by immunoaffinity enrichment combined with MALDI mass spectrometry is rapid, does not rely on external purification steps, can be automated, and consumes amounts of antibody comparable with standard ELISA (Pompach P et al Clin. Chem. 2016).



Left-MS *spectra of haptoglobin subunits enriched using functionalized MALDI surfaces. Spectra colors are based on the phenotype: Hp 1-1 (blue), Hp 2-1 (green), and Hp 2-2 (yellow).* ***Right- Spectrum*** *of transferrin isolate from whole blood of healthy patient (A) and alcoholic patient (B). Transferrin isoforms TeST, DST, and AST differ in N-glycosylation site occupancy by biantennary doubly sialylated glycans.*

The haptoglobin success suggested to an idea to extend the portfolio of available clinical test for detection and quantification of carbohydrate-deficient transferrin. Transferrin is the liver secreted glycoprotein important for iron transport. Severe alcohol consumption leads to alterations in glycosylation of transferrin. In this study, combination of surfaces modified with antitransferrin antibody and MALDI mass spectrometry were used for quantification of transferrin glycoforms in patients with alcohol abuse (see above). A sample cohort of 186 patients was analysed and correlated with capillary electrophoresis from Sebia. The results demonstrate that the CDT assay based on the MALDI chips can be validated for use in clinical practice. The main advantages of the presented approach are that the calibration-free MALDI chip work flow is fast, does not require sample separation, is more robust with respect to sample quality, and can be automated for high-throughput analysis (Darebna P. et al, Clin. Chem. 2018).

A potential of an ambient ion-landing technique to effectively immobilize enzymes on conductive supports for MALDI mass spectrometry analyses of reaction products enables to use this chip technology for in situ time-limited proteolysis of proteins and protein–ligand complexes to monitor their structural changes under different conditions. Three serine proteases, trypsin, chymotrypsin and subtilisin A, were immobilized and successfully used for monitoring of protein structural changes. The data from limited proteolysis using MALDI chips fits to known or predicted protein structures. The results show that functionalized MALDI chips are sensitive, robust, and fast and might be automated for general use in the field of structural biology (Rosulek M. et al., Catalyst 2019).



Limited tryptic 15-minute proteolysis of aMyo and hMyo forms. *Butterfly plot visualization of the limited proteolysis experiment for aMyo and hMyo forms. The cleavage sites 42, 133, and 145 were preferentially cleaved in hMyo, whereas sites 45 and 96 were compromised. (b) The structure of hMyo with preferred cleavage sites is highlighted in green. The highlighted regions in red color, which include residues 45 and 96, are located close to the heme binding region and are less accessible for protease when heme is present. Heme molecule is highlighted in blue.*

Research activity and characterisation of the main scientific results

The group will largely continue the research in the fields that are already established and extend the current approaches methodically. Among the results outlined below, we mostly value those original science reports qualified among the ISI Highly Cited Papers, that were either exclusively authored by group members or led by the group (Urbanová et al. 2015, Brabcová et al. 2016, Žifčáková et al. 2016) and the recent paper describing global fungal diversity and its response to climate (Větrovský et al. 2019).

Factors shaping the composition and dynamics of microbial communities in the environment

This subject remain to be studied the laboratory, although the involvement of the microbiome in ecosystem processes receives much more attention. The factors shaping the composition of microbial communities were studied in the forest soil context showing the effects of vegetation, chemistry and vertical stratification on the composition of communities of bacteria and fungi (Bahnmann et al. 2018, López-Mondéjar et al. 2015, Urbanová et al. 2015). Deadwood represented another habitat where microbiome composition was studied. Here, the chemistry and length of decay, as well as host and microclimate effects were identified as the drivers of microbial succession (Baldrian et al. 2016, Krah et al. 2018, Tláskal et al. 2017). Grassland plant-associated and soil microbiomes were characterized in the context of plant diversity gradient (Navrátilová et al. 2019). Since forest soil are in the focus of the team for a long time, several review papers considered microbial diversity in this ecosystem, pointing out their complexity and habitat-specificity (Baldrian et al. 2017, Lladó et al. 2017).

The analysis of microbiomes always limits the explanatory power to the environment that was sampled and that is typically spatially limited. To overcome the limitations of single studies, our group undertook the effort to catalogue, collect and analyse all reports of fungal community composition in terrestrial ecosystems that were not subject to experimental manipulation. The analysis involved >400 papers published until 2017 and collected data from >5500 locations worldwide. The resulting dataset was parsed with climate data and utilised for the analysis of global patterns of fungal diversity and prediction of environmental niches of >450 fungal species. The results show that, unlike plants and animals, diversity of fungi peaks outside tropical regions. In addition, niche modelling showed that climate has decisive importance for global fungal distribution, its effects being much larger than those of vegetation and soil properties. Interestingly, symbiotic root-associated fungi show much narrower climatic niche than plant pathogens and are thus more vulnerable to climate change. The resulting paper of Větrovský et al. (2019) attracted immediate attention: by mentions in social media, it belongs to top 10% papers in Nature Communications, and results appeared in the Czech television, press and popular science journals.

Selected results:

Bahnmann, B., Mašínová, T., Halvorsen, R., Davey, M.L., Sedlák, P., Tomšovský, M., Baldrian, P., 2018. Effects of oak, beech and spruce on the distribution and community structure of fungi in litter and soils across a temperate forest. Soil Biology & Biochemistry 119, 162-173.
* Baldrian, P., 2017. Forest microbiome: Diversity, complexity and dynamics. FEMS Microbiology Reviews 41, 109-130. (ISI Highly Cited Paper)
Baldrian, P., Zrůstová, P., Tláskal, V., Davidová, A., Merhautová, V., Vrška, T., 2016. Fungi associated with decomposing deadwood in a natural beech-dominated forest. Fungal Ecology 23, 109-122.
Krah, F.-S., Seibold, S., Brandl, R., Baldrian, P., Müller, J., Bässler, C., 2018. Independent effects of host and environment on the diversity of wood-inhabiting fungi. Journal of Ecology 106, 1428-1442.
Lladó, S., López-Mondéjar, R., Baldrian, P., 2017. Forest soil bacteria: Diversity, involvement in ecosystem processes, and response to global change. Microbiology and Molecular Biology Reviews 81, 00063-00016.
López-Mondéjar, R., Voříšková, J., Větrovský, T., Baldrian, P., 2015. The bacterial community inhabiting temperate deciduous forests is vertically stratified and undergoes seasonal dynamics. Soil Biology & Biochemistry 87, 43-50.
Mašínová, T., Bahnmann, B.D., Větrovský, T., Tomšovský, M., Merunková, K., Baldrian, P., 2017. Drivers of yeast community composition in the litter and soil of a temperate forest. FEMS Microbiology Ecology 93, fiw223.
Navrátilová, D., Tláskalová, P., Kohout, P., Dřevojan, P., Fajmon, K., Chytrý, M., Baldrian, P., 2019. Diversity of fungi and bacteria in species-rich grasslands increases with plant diversity in shoots but not in roots and soil. FEMS Microbiology Ecology 95, fiy208.
Tláskal, V., Zrůstová, P., Vrška, T., Baldrian, P., 2017. Bacteria associated with decomposing dead wood in a natural temperate forest. FEMS Microbiology Ecology 93, fix157.
* Urbanová, M., Šnajdr, J., Baldrian, P., 2015. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. Soil Biology & Biochemistry 84, 53-64. (ISI Highly Cited Paper)
Větrovský, T., Kohout, P., Kopecký, M., Machac, A., Man, M., Bahnmann, B.D., Brabcová, V., Choi, J., Meszárošová, L., Human, Z.R., Lepinay, C., Lladó, S., Lopez-Mondejar, R., Martinovic, T., Mašínová, T., Morais, D., Navrátilová, D., Odriozola, I., Štursová, M., Švec, K., Tláskal, V., Urbanová, M., Wan, J., Žifčáková, L., Howe, A., Ladau, J., Peay, K.G., Storch, D., Wild, J., Baldrian, P., 2019. A meta-analysis of global fungal distribution reveals climate-driven patterns. Nature Communications 10, 5142.

Involvement of fungi and bacteria in ecosystem processes

The analysis of fungal and bacterial contribution to ecosystem processes was addressed in three complementary ways: in the first one, these taxonomic groups were individually focused and their genomic features and their expression were studied in laboratory setting by the analysis of environmental isolates. In this way, we have distinguished the ecological and taxonomic factors that shape the production of decomposition-related enzymes by fungi (Eichlerová et al. 2015) and later delimited the nutritional niche of yeasts as a specific group of fungi with adaptations to unicellularity and shared features with bacteria (Mašínová et al. 2018).

The topic of bacterial functioning in forest soils was covered by the postdoctoral fellows Salvador Lladó and Rubén López-Mondéjar with the main aim to characterize

the set of bacterial isolates that represent highly abundant taxa in forest litter and soil and those taxa that are able to decompose lignocellulose. The results include the characterization of bacteria, participating in lignocellulose decomposition and their genomic adaptation and expression (López-Mondéjar et al. 2016, 2019).

The second approach was the analysis of microbes participating in individual ecosystem processes. The isolation of dominant forest soil bacteria allowed us, for the first time for the soil environment, to describe the transcriptional activity of individual bacterial taxa. This approach led to the delineation of the “opportunistic” and “decomposer” guilds of bacteria (Lladó et al. 2019) and supplemented the so-far accumulated knowledge on bacterial contribution to soil processes (Lladó et al. 2018). Another topic of focus was the decomposition of fungal biomass in soil. The microbial communities, participating in this process, were described and the decomposing biomass of fungi was demonstrated to be a hotspot of bacterial activity (Brabcová et al. 2016). Later, it was recorded that bacterial communities decomposing mycelia are substrate specific and the rate of mycelium decomposition was shown to be strongly dependent on nitrogen content (Brabcová et al. 2018). Decomposition of cellulose was a target of a study by a guest in the laboratory (Pathan et al. 2018). In a complex experimental setting, the whole flow of carbon in the decomposer food web was characterized using stable-isotope labelled biomass of plants, fungi and bacteria, demonstrating the complexity of metabolic dependencies and C flow (López-Mondéjar et al. 2018).

The third approach to characterize microbial ecosystem processes utilized the whole metatranscriptomes of forest soil to track the activity of all members of the microbiome. The experiments asked the question about the seasonal changes in microbial transcription in temperate forests where the seasonal activity of roots was predicted as the most important driver. We have succeeded in publishing the first results of soil transcriptome analysis that considered environment heterogeneity by sampling replicated sites and demonstrated that the activity of fungi and bacteria differs between seasons and the ectomycorrhizal root symbionts are mostly active only when the roots of trees supply them with photosynthetic C (Žifčáková et al. 2016). In a follow-up study, we have shown that carbon compounds utilized by microorganisms differ between summer and winter and quantified the share of fungi and bacteria on biopolymer decomposition in forest litter and soil (Žifčáková et el. 2017).

After these results, we have succeeded to apply for a funding from the Department of Energy of the USA to run the metatranscriptome, metagenome, metametabolome and metaproteome analysis of seasonality at the plant root-soil interface utilizing the infrastructure of the Joint Genome Institute and Environmental Molecular Sciences Laboratory. The project compares C and N cycling across seasons in soils of Central Europe and Norway that differ in N availability. The international cooperative project is led by P. Baldrian and includes the participation of Oslo University (Havard Kauserud), INRA Nancy (Francis Martin), Iowa State University (Adina Howe) and University of Greifswald (Katharina Riedel). At this moment, the sampling and analyses in the US laboratories are finished and the data are being analysed. Initial results demonstrate habitat specificity and seasonality of both N and C cycling and the context dependent metabolome composition affecting microbial communities.

Selected results:

* Brabcová, V., Nováková, M., Davidová, A., Baldrian, P., 2016. Dead fungal mycelium in forest soil represents a decomposition hotspot and a habitat for a specific microbial community. New Phytologist 210, 1369-1381. (ISI Highly Cited Paper)
Brabcová, V., Štursová, M., Baldrian, P., 2018. Nutrient content affects the turnover of fungal biomass in forest topsoil and the composition of associated microbial communities. Soil Biology & Biochemistry 118, 187-198.
Eichlerová, I., Homolka, L., Žifčáková, L., Lisá, L., Dobiášová, P., Baldrian, P., 2015. Enzymatic systems involved in decomposition reflects the ecology and taxonomy of saprotrophic fungi. Fungal Ecology 13, 10-22.
Lladó, S., López-Mondéjar, R., Baldrian, P., 2017. Forest soil bacteria: Diversity, involvement in ecosystem processes, and response to global change. Microbiology and Molecular Biology Reviews 81, 00063-00016.
Lladó, S., Větrovský, T., Baldrian, P., 2019. Tracking of the activity of individual bacteria in temperate forest soils shows guild-specific responses to seasonality. Soil Biology and Biochemistry 135, 275-282.
López-Mondéjar, R., Algora, C., Baldrian, P., 2019. Lignocellulolytic systems of soil bacteria: A vast and diverse toolbox for biotechnological conversion processes. Biotechnology Advances 37, 107374.
López-Mondéjar, R., Brabcová, V., Štursová, M., Davidová, A., Jansa, J., Cajthaml, T., Baldrian, P., 2018. Decomposer food web in a deciduous forest shows high share of generalist microorganisms and importance of microbial biomass recycling. ISME Journal 12, 1768-1778.
López-Mondéjar, R., Zühlke, D., Větrovský, T., Becher, D., Riedel, K., Baldrian, P., 2016. Decoding the complete arsenal for cellulose and hemicellulose deconstruction in the highly efficient cellulose decomposer *Paenibacillus* O199.
Mašínová, T., Yurkov, A., Baldrian, P., 2018. Forest soil yeasts: Decomposition potential and the utilization of carbon sources. Fungal Ecology 34, 10-19.
Pathan, S.I., Žifčáková, L., Ceccherini, M.T., Pantani, O.L., Větrovský, T., Baldrian, P., 2017. Seasonal variation and distribution of total and active microbial community of β-glucosidase encoding genes in coniferous forest soil. Soil Biology and Biochemistry 105, 71-80.
* Žifčáková, L., Větrovský, T., Howe, A., Baldrian, P., 2016. Microbial activity in forest soil reflects the changes in ecosystem properties between summer and winter. Environmental Microbiology 18, 288-301. (ISI Highly Cited Paper)
Žifčáková, L., Větrovský, T., Lombard, V., Henrissat, B., Howe, A., Baldrian, P., 2017. Feed in summer, rest in winter: microbial carbon utilization in forest topsoil. Microbiome 5, 122.

Ecosystem development and dynamics of ecosystem processes

The collaborative projects headed by Prof. J. Frouz from Charles University, Prague, focused on the drivers of microbial community functioning in the developing ecosystems of various post-mining sites and the development of forests in Papua New Guinea that were switched to agricultural lands. In both cases, our team was responsible for the microbiome analysis. We have provided a comprehensive description of soil microbiome development along primary succession on post-mining lands identifying changes in soil chemistry and vegetation as primary drivers of microbial colonization and succession (Harantová et al. 2017). On the same sites, we also showed importance of biotic interactions in structuring root microbiome (Kolaříková et al. 2017). For the Papua study, the results indicate that native agriculture

ensures the permanent existence of symbiotic fungi in the cycle of rainforest – agriculture – regrowth which aids the cyclic utilization of lands in this tropical context (Kukla et al. 2019). P. Baldrian collaborated with the group of J.D. van Elsas on the analysis of marine to land transition on maritime sand banks that focused fungal communities. Plant arrival was identified as a decisive turning point of fungal community succession (Dini-Andreotte et al. 2016). The dynamics of forest ecosystems was studied in a management context by following microbial activity and fungal community after clearcutting. The results indicate rapid restructuring of fungal community and the reduction of decomposition as a result of the reduction of the stand primary productivity (Kohout et al. 2018). Dynamics of forest ecosystems was also an important topic of invited review papers (Baldrian et al. 2017a,b).

Selected results:

* Baldrian, P., 2017a. Forest microbiome: Diversity, complexity and dynamics. FEMS Microbiology Reviews 41, 109-130. (ISI Highly Cited Paper)
Baldrian, P., 2017b. Microbial activity and the dynamics of ecosystem processes in forest soils. Current Opinion in Microbiology 37, 128-134.
Dini-Andreote, F., Pylro, V.S., Baldrian, P., Van Elsas, J.D., Salles, J.F., 2016. Ecological succession reveals potential signatures of marine-terrestrial transition in salt marsh fungal communities. ISME Journal 10, 1984-1997.
Harantova, L., Mudrak, O., Kohout, P., Elhottova, D., Frouz, J., Baldrian, P., 2017. Development of microbial community during primary succession in areas degraded by mining activities. Land Degradation & Development 28, 2574-2584.
Kohout, P., Charvátová, M., Štursová, M., Mašínová, T., Tomšovský, M., Baldrian, P., 2018. Clearcutting alters decomposition processes and initiates complex restructuring of fungal communities in soil and tree roots. ISME Journal 12, 692-703.
Kolaříková, Z., Kohout, P., Krüger, C., Janoušková, M., Mrnka, L., Rydlová, J., 2017. Root-associated fungal communities along a primary succession on a mine spoil: Distinct ecological guilds assemble differently. Soil Biology and Biochemistry 113, 143-152.
Kukla, J., Whitfeld, T., Cajthaml, T., Baldrian, P., Veselá-Šimáčková, H., Novotný, V., Frouz, J., 2019. The effect of traditional slash-and-burn agriculture on soil organic matter, nutrient content, and microbiota in tropical ecosystems of Papua New Guinea. Land Degradation & Development 30, 166-177.

Interactions of microorganisms with invertebrates and plants

The analysis of the interactions between microbes and invertebrates was continued in collaboration with Yale University, demonstrating the importance of biotic interaction on decomposition in a changing climate (Crowther 2015). In addition, plant-invertebrate interactions were studied in the context of deadwood decomposition in a project led by German group (Jörg Müller and Sebastian Seibold, Würzburg University, Claus Bässler TU Munich) focusing several questions including the role of insects in fungal dispersal (Seibold et al. 2016, 2019, Veselská et al. 2019). The project headed by Prof. J. Sobotnik from the Czech University of Life Sciences focused on the exploration of microbiomes associating with termites in tropical regions (results to be published in future). Considering plant-microbe interaction, our group has demonstrated the decisive role of plants for the shaping of fungal and bacterial communities in litter and soil and reported the interaction between plants and fungi to

be stronger than between plants and bacteria (Urbanová et al. 2015). We have also analysed the question of the link between plant and microbial diversity in a grassland system with a wide gradient of plant diversity. The diversity of bacteria and fungi was found to increase with plant diversity on aboveground tissues, but not on roots and in soil (Navrátilová et al. 2019). Plant microbial interactions were also studied in the environment of a developing community in a limestone quarry with a focus on plant-soil feedback. This project was led by the Institute of Botany and results will be published later. Our team was responsible for the analysis of the microbiome-associated part of the feedback. The exploration of the effects of plants in fungal invasions was initiated by P. Kohout.

Selected results:

* Urbanová, M., Šnajdr, J., Baldrian, P., 2015. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. Soil Biology & Biochemistry 84, 53-64. (ISI Highly Cited Paper)
Crowther, T.W., Thomas, S.M., Maynard, D.S., Baldrian, P., Covey, K., Frey, S.D., Van Diepen, L.T.A., Bradford, M.A., 2015. Biotic interactions mediate soil microbial feedbacks to climate change. Proceedings of the National Academy of Sciences of the United States of America 112, 7033-7038.
Development of tools to study microbial communities and processes in soils
Navrátilová, D., Tláskalová, P., Kohout, P., Dřevojan, P., Fajmon, K., Chytrý, M., Baldrian, P., 2019. Diversity of fungi and bacteria in species-rich grasslands increases with plant diversity in shoots but not in roots and soil. FEMS Microbiology Ecology 95, fiy208.
Seibold, S., Bässler, C., Baldrian, P., Reinhard, L., Thorn, S., Ulyshen, M.D., Weiß, I., Müller, J., 2016. Dead-wood addition promotes non-saproxylic epigeal arthropods but effects are mediated by canopy openness. Biological Conservation 204, 181-188.
Seibold, S., Müller, J., Baldrian, P., Cadotte, M.W., Šťursová, M., Biedermann, P.H.W., Krah, F.-S., Bässler, C., 2019. Fungi associated with beetles dispersing from dead wood – Let's take the beetle bus! Fungal Ecology 39, 100-108.
Veselská, T., Skelton, J., Kostovčík, M., Hulcr, J., Baldrian, P., Chudíčková, M., Cajthaml, T., Vojtová, T., Garcia-Fraile, P., Kolařík, M., 2019. Adaptive traits of bark and ambrosia beetle-associated fungi. Fungal Ecology 41, 165-176.

Development of methods to study microbiome composition and function

The focus of the group on fungal community exploration led to the development of novel approaches to their molecular identification and demonstrated the advantages of such alternative barcoding techniques for the estimation of fungal diversity (Větrovský et al. 2016). Generally, utilization of high-throughput methods of fungal community analysis inspired collaborative papers comparing the reporting the advantages of different methods and making recommendations for their further use (Anslan et al. 2018, Nilsson et al. 2019). The experience with the identification of active fraction of the fungal community resulted in the publication of a protocol (Větrovský et al. 2016b). Finally, the bioinformatics treatment of large data is aided by the development of novel tools to analyse sequencing results of amplicon-based metagenomics that increase the comfort of users (Morais et al. 2018, Větrovský et al. 2018). Especially the developed bioinformatics pipeline SEED / SEED2 received

considerable attention and is frequently utilised as demonstrated by 140 citations to the software papers.

Selected results:

Anslan, S., Nilsson, R.H., Wurzbacher, C., Baldrian, P., Tedersoo, L., Bahram, M., 2018. Great differences in performance and outcome of high-throughput sequencing data analysis platforms for fungal metabarcoding. Mycokeys, 29-40.
Morais, D.K., Roesch, L.F.W., Redmile-Gordon, M., Santos, F.G., Baldrian, P., Andreote, F.D., Pylro, V.S., 2018. BTW-Bioinformatics Through Windows: an easy-to-install package to analyze marker gene data. Peerj 6, e5299.
* Nilsson, R.H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nature Reviews Microbiology 17, 95-109. (ISI Highly Cited Paper)
Větrovský, T., Baldrian, P., Morais, D., 2018. SEED 2: a user-friendly platform for amplicon high-throughput sequencing data analyses. Bioinformatics 34, 2292-2294.
Větrovský, T., Kolařík, M., Žifčáková, L., Zelenka, T., Baldrian, P., 2016a. The *rpb2* gene represents a viable alternative molecular marker for the analysis of environmental fungal communities. Molecular Ecology Resources 16, 388-401.
Větrovský, T., Štursová, M., Baldrian, P., 2016b. Fungal communities in soils: Soil organic matter degradation, Methods in Molecular Biology, pp. 89-100.

Applied research and the research for practice

Although the bulk of the research output of the laboratory is basic research, some results have a potential for future development in the frames of the applied research. It is namely the characterization of bacterial strains from the soil environment that produce biotechnologically relevant enzymes and the effort to utilize citizen science (see below) to screen, isolate and pre-select biotechnologically relevant fungi. In addition, the research on management effects of ecosystems takes into consideration the needs of the stakeholders and the results are reported to them – such as the exploration of drivers of microbial diversity and communities in the National Park Bohemian Forest, National Park Bavarian Forest, Protected Landscape Area Bílé Karpaty and the Zofin Natural Reserve. The results may aid the development of guidelines for the protection of nature. The results of the exploration of anthropogenic habitats like spoil deposits are available to managers of these area and help them to choose the best practice for the treatment of disturbed sites. Last but not least, the analysis of the effects of climate on global distribution of fungi opens the questions about the potential change of fungal communities and fungal-mediated ecosystem processes in the face of the global change. The observation that symbiotic root-associated fungi essential for agricultural and forestry production are climate-sensitive while plant pathogens are climate-resistant (Větrovský et al. 2019) focuses the attention to the need to take measures to protect ecosystem functions and ensure food safety.

Research activity and characterisation of the main scientific results

Herein described team forms the research part of the Centre of Nanobiology and Structural Biology (CNBS) which is located in Nové Hrady as the detached centre of Institute of Microbiology. The whole Nove Hrady team, including its sub-teams (the individual groups/labs), is evaluated as one unit.
In the evaluated period, the team described here published 53 impacted publications. Thanks to that, scientists from Nove Hrady are regularly invited to speak at international conferences and symposia, get invitations to contribute review papers, book chapters and invited papers. CNSB is regularly visited by foreign scientists and participants of international internship programs, for example each year the institute hosts up to 10 students from Princeton University and other American universities. Scientists in CNSB use methods from bioinformatics, molecular biology, microscopy, and molecular modelling and structure determination. They work in the field which so called structural systems biology on a molecular, cell, and tissue level that provides knowledge on the molecular level of structural system elements, their principal metabolic and control pathways, identifies links between these elements and thus describes the structure of biological systems.

The research team has its roots since 2002 when the laboratory of high-performance computing was established in Nove Hrady (R. Ettrich, M. Kuty) and started to cooperate with the newly established laboratory of crystallogenesis (I. Kuta Smatanova). The research group grew quickly and the demands for molecular biology on a high level lead to engagement of E. Csefalvay in 2005. In the following years the strength of the research team formed by the two groups was in the close collaboration of the two laboratories that allow a very complex research approach using a broad range of methods with a synthesis of theoretical and experimental protein research. In 2008, the two groups were joined by Josef Lazar, who established a laboratory of cell biology and focused on the development of two-photon polarization microscopy. In March 2011 David Reha from Essex University joined the team to establish a knowledge base for QM/MM hybrid methods in computational biology.
In January 2014, Jost Ludwig moved his lab from Bonn University to Nove Hrady and joints the team, bringing his expertise on functional analysis of ion channels and transporters, cation transport in yeast (Saccharomyces Cerevisiae). The research team is a founding member of the Czech Infrastructure for Systems Biology C4SYS. Several of the sub-teams were contributing services to C4SYS and were involved in its implementation. Since 2016 the team in Nove Hrady moved to the Institute of Microbiology as its detached centre – Center fo Nanabiology nad Structural Biology.

The research team has highly ambitious aims with regard to develop new methods in molecular systems biology and its application to systems of a common interest of hot topic systems both experimentally and computationally.

Currently four research groups are closely collaborating within the research team in Nove Hrady:

1. Structure and Function of Proteins and Computational Biology (Dr. David Řeha) [Lab 193]

2. Crystallogenesis and Biomolecular Crystallography (assoc. Prof. Ivana Kuta Smatanova) [Lab 192]
3. Advanced Optical Microscopy and cell biology (Dr. Josef Lazar) [Lab 194]
4. Membrane Physiology and Bioenergetics (Dr. Jost Ludwig) [Lab 195]

1. Structure and Function of Proteins and Computational Biology (Dr. David Řeha)

During the evaluating period this group operated as the laboratory of Structure and Function of Proteins (Lab 193) headed by Prof. Rudiger H. Ettrich, who was leading this research group until August 2018 and who was also the head of CNSB. The Lab 193 has two sub-groups; Computational Biology led by Dr. David Řeha and Molecular Liquids led by Dr. Babak Minofar. Since 2018, after leaving of Prof. Ettrich, the Lab 193 is now led by Dr. David Řeha and Dr. Babak Minofar becomes the head of the whole Nove Hrady team (CNSB).
The research groups are interested mainly in the relationship between structure and function of proteins, dynamic changes related to functional processes on the level of proteins and the mutual interaction of cofactors and subunits in protein complexes. Additionally, the group focusses also on the interaction of protein and the other biomolecules with various solvents and molecular liquids. The research approach is complex using various methods of protein research with a synthesis of theoretical and experimental methods. Quantum chemical and semi-empirical calculations and molecular modelling methods are combined with mostly spectroscopic, thermodynamic and crystallographic methods and methods of protein structure determination.

a) One focus in the evaluated period is based on the long experiences with computational modelling and molecular dynamics simulations of ion channel. One of our main research activities focused on the STIM-Orai system. The stromal interaction molecule (STIM) is a protein located at the level of the membrane from the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR). This protein has a double function in the cellular life. The N-terminal, bathing in the lumen of the ER, has the ability to probe the calcium level within the ER thanks to an EF-hand motif. On the other side of the membrane, the cytosolic part is responsible for activating target calcium-channels in the plasma membrane depending on the calcium level within the ER. The most common ion-channel associated with STIM is the calcium channel Orai. When STIM is associated together to Orai, they form the so-called SOC (store operated calcium) channel. This project exclusively relies on the computational expertise from the team based in Nové Hrady. The focus of the team can be divided in two major axes of research. The first axis being around the Orai protein while the second axis, more recent, is dedicated to STIM.
Concerning to Orai, the closed state of the channel has been resolved since 2012. However, it is only recently, in 2019, that the first structures from the open state were available. Yet, the resolution for these structures is quite low. Furthermore, although both structures of the channel in its open and closed state were resolved, little is known about how STIM is able to convert the channel from a quiescent state to a leaky state. Our work consisted, first, on the identifying key region involved in the gating of calcium ions. Further on, and based on our acquired knowledge on the channel, simulation of gain or loss-of-function mutations discovered by our

experimental partners in Linz were being performed. The driving motive was to understand the mechanism lying behind the activation of the channel. More specifically, we rely on atomistic simulations to decipher the unfolding of events and changes in intramolecular interactions driving the channel from the closed to the open state. The resolved available structures are obtained from drosophila and lack several domains in the extra and intracellular loops. Our first step in this direction was to use homology modeling to build a structure of the human Orai1 channel based on the drosophila channel. The simulations using this newly forged model helped to identify a calcium accumulating region at the entry of the pore increasing the conductance of the channel. This region is present in the extracellular loops and was absent in the original crystallographic structure. Our initial 3D model was then deposited in the Model Archive (http://modelarchive.org, accession code ma-akdjp doi:10.5452/ma-akdjp) and the results were published in 2015. Based on this initial success, we extended our protocol to the other two members of the human Orai family, namely Orai2 and Orai3. Our reconstructed model was now put to the test to investigate other loops, this time present in the cytosol, and identified by our partner in Linz to be involved in the binding to STIM1 and further on to the activation of the channel. Our simulations were able to link the decrease in activation by STIM1 observed experimentally when a portion of the protein (N-terminus domain forming the conducting pore) was deleted. MD simulations helped to reveal the existence of fine-tuned interactions between the loop2 and the N-terminus responsible for a different level of gating activation in the different Orai isoforms. We published the outcomes of this work in 2018. As previously stated, *in-silico* mutagenesis were carried out alongside experiments to explore the gating mechanism of Orai. The Linz group systematically screened for dysfunctional Orai1 mutants derived from large-scale cancer genomics data sets and identified constitutively active and Ca^{2+} selective mutations. From the simulations of these mutations, we elaborated an Orai1 gating model involving the rotation of the helices forming the pore centered on phenylalanines. Upon activation, the pore dilates and these residues rotate away allowing a flow a water molecule within the channel and thereby reducing hydrophobic and electrostatic barriers for calcium entry. Hereby, we showed that a hydrophobic domain, coupled together with a basic rich residues region located below, are playing a key role in gating. The results were published in 2017. [Frischauf I., Zayats V., Deix M., Hochreiter A., Jardin I., Muik M., Lackner B., Svobodova B., Pammer T., Litvinukova M., Sridhar A. A., Derler I., Bogeski I., Romanin C., Ettrich R. H. & Schindl R. A calcium-accumulating region, CAR, in the channel Orai1 enhances Ca2+ permeation and SOCE-induced gene transcription. *Science Signaling* (2015) **8**, doi:10.1126/scisignal.aab1901].

The association of STIM and Orai channels involved in store-operated calcium entry takes place within lipid rafts rich in cholesterol. Also, it has been shown that the association of STIM and TRPC1 proteins within lipids rafts is regulated by the ER calcium stores. We focused on the role of cholesterol in the regulation of STIM1-mediated Orai1 currents. The experimental results gained in the Linz group demonstrate that chemically induced cholesterol depletion enhanced store-operated Ca2+ entry and Orai1 currents. We were able to publish these results in 2016 which hint on the role of cholesterol in the regulation of the Orai1 channel and suggest the possibility of direct cholesterol binding to the transmembrane region of the channel, however, leave open the question where the potential cholesterol-binding site would be localized. [Derler I., Jardin I., Stathopulos P. B., Muik M., Fahrner M., Zayats V., Pandey S. K., Poteser M., Lackner B., Absolonova M., Schindl R., Groschner K.,

Ettrich R., Ikura M. & Romanin C. Cholesterol modulates Orai1 channel function. *Science Signaling* (2016) **9**, doi:10.1126/scisignal.aad7808]
Following our work on Orai, our team naturally started to work on STIM1 as well. In a similar fashion to the mutagenesis study performed on Orai1, our partner in Graz screened for dysfunctional STIM1 mutants derived from large-scale cancer genomics data sets and identified mutation leading to constitutively active mutants. STIM is rather a complex protein possessing domains present either in the luminal domain of the ER or inside the cytosol. Each of these domains having specific roles. We focused here on the domain present inside the ER responsible for Ca2+ sensing. Several of the mutation identified was located on a portion of the luminal domain formed by a pair of EF-hands. Our simulations revealed that the mutations responsible for the activation of STIM destabilized the EF-hands inducing partial unfolding of the luminal domain. We hypothesized that this denaturation is linked to the agglomeration seen during activation. Our results were published in 2019.

·Fahrner M., Pandey S. K., Muik M., Traxler L., Butorac C., Stadlbauer M., Zayats V., Krizova A., Plenk P., Frischauf I., Schindl R., Gruber H. J., Hinterdorfer P., Ettrich R., Romanin C. & Derler I. Communication between N terminus and loop2 tunes Orai activation. *Journal of Biological Chemistry* (2018) **293**, 1271-1285, doi:10.1074/jbc.M117.812693.
·Bonhenry D., Schober R., Schmidt T., Waldherr L., Ettrich R. H. & Schindl R. Mechanistic insights into the Orai channel by molecular dynamics simulations. *Seminars in Cell & Developmental Biology* (2019) **94**, 50-58, doi:10.1016/j.semcdb.2019.01.002.
·Schober R., Bonhenry D., Lunz V., Zhu J. H., Krizova A., Frischauf I., Fahrner M., Zhang M. Q., Waldherr L., Schmidt T., Derler I., Stathopulos P. B., Romanin C., Ettrich R. H. & Schindl R. Sequential activation of STIM1 links Ca2+ with luminal domain unfolding. *Science Signaling* (2019) **12**, doi:10.1126/scisignal.aax3194.

b) Another project was focused on cleavage and translocation by Type I restriction-modification complexes. This project started with resolving the crystal structure of the motor subunit of EcoRI that we published in Nature Structural&Molecular Bilogy in 2009, and is aimed at providing fundamental knowledge about restriction enzymes and translocases that can help to describe the function and evolution of cellular DNA defense and repair systems. Furthermore, understanding the cooperation of translocase and nuclease activities could provide a tool to regulate the nuclease activities as a potential therapeutic target. The Type I RM enzymes have been suggested as potential tools for assembly of nano-machines because they provide a molecular motor that would not cut its own DNA track during translocation. The results during the evaluated period were published in the following papers:
·Csefalvay E., Lapkouski M., Guzanova A., Csefalvay L., Baikova T., Shevelev I., Bialevich V., Shamayeva K., Janscak P., Smatanova I. K., Panjikar S., Carey J., Weiserova M. & Ettrich R. Functional Coupling of Duplex Translocation to DNA Cleavage in a Type I Restriction Enzyme. *Plos One* (2015) **10**, doi:10.1371/journal.pone.0128700.
·Grinkevich P., Iermak I., Luedtke N. A., Mesters J. R., Ettrich R. & Ludwig J. pHluorin-assisted expression, purification, crystallization and X-ray diffraction data analysis of the C-terminal domain of the HsdR subunit of the Escherichia coli type I restriction-modification system EcoR124I. *Acta Crystallographica Section F-Structural Biology Communications* (2016) **72**, 672-676, doi:10.1107/s2053230x16011626.

·Bialevich V., Sinha D., Sharnayeva K., Guzanova A., Reha D., Csefalvay E., Carey J., Weiserova M. & Ettrich R. H. The helical domain of the EcoR1241 motor subunit participates in ATPase activity and dsDNA translocation. *Peerj* (2017) **5**, doi:10.7717/peerj.2887.
·Grinkevich P., Sinha D., Iermak I., Guzanova A., Weiserova M., Ludwig J., Mesters J. R. & Ettrich R. H. Crystal structure of a novel domain of the motor subunit of the Type I restriction enzyme EcoR124 involved in complex assembly and DNA binding. *Journal of Biological Chemistry* (2018) **293**, 15043-15054, doi:10.1074/jbc.RA118.003978.
·Grinkevich P., Sinha D., Iermak I., Guzanova A., Weiserova M., Ludwig J., Mesters J. R. & Ettrich R. H. Crystal structure of a novel domain of the motor subunit of the Type I restriction enzyme EcoR124 involved in complex assembly and DNA binding. *Journal of Biological Chemistry* (2018) **293**, 15043-15054, doi:10.1074/jbc.RA118.003978.

c) Another long term focus of the laboratory is on allostery effect in biomolecules. A controversial prediction of the famous allostery model of Monod, Wyman, and Changeux is that constraints imposed on protein subunits by multimerization are relaxed by ligand binding, but with the conservation of symmetry in partially-liganded states. Interpretation of thermodynamic ligand-binding data through the lens of molecular dynamics simulation has led to the structural and energetic description of such a state for the hexameric Escherichia coli arginine repressor, which displays strong negative cooperativity of L-arginine binding. The results indicate that partially-liganded states can be structurally symmetric despite their conceptual asymmetry. The symmetric relaxed state is visualized as a multimer with all subunits anchored near the center, and with motions transferred to the periphery of the assembly. Thus, even during the sequential filling of binding sites, symmetry can be maintained by exploiting the dynamics of the assembly and the distributed nature of its cohesive free energy. This elegantly simple and probably ancient molecular mechanism of allostery is described for the Escherichia coli arginine repressor ArgR, the master feedback regulator of transcription in L-arginine metabolism.
Molecular dynamics simulations with ArgRC, the hexameric domain that binds L-arginine with negative cooperativity, reveal that conserved arginine and aspartate residues in each ligand-binding pocket promote rotational oscillation of apoArgRC trimers by engagement and release of hydrogen-bonded salt bridges. The results thus offer the first opportunity to describe in structural and thermodynamic terms the symmetric relaxed state predicted by the concerted allostery model of Monod, Wyman, and Changeux, revealing that this state is achieved by exploiting the dynamics of the assembly and the distributed nature of its cohesive free energy. The ArgR example reveals that symmetry can be maintained even when binding sites fill sequentially due to negative cooperativity, which was not anticipated by the Monod, Wyman, and Changeux model. The molecular mechanism identified neither specifies nor requires a pathway for transmission of the allosteric signal through the protein, and it suggests the possibility that binding of free amino acids was an early innovation in the evolution of allostery.

d) Another project was the study of the reaction center of Photosystem II using MD simulations and QM/MM calculations. Photosystem II (PSII) is a multi-subunit pigment-protein complex and is one of several protein assemblies that function cooperatively in photosynthesis in plants and cyanobacteria. As more structural data

on PSII become available, new questions arise concerning the nature of the charge separation in PSII reaction centre (RC). We have focused on the dynamics of the reaction center of Photosystem II upon charge separation and subsequent charge transfer steps. The conformational behavior of protein and the cofactors directly involved in the charge separation were studied by MD simulations and QM/MM calculations. Our study identified the most likely mechanism of the proton-coupled reduction of plastoquinone QB. After the charge separation and the first electron transfer to QB, the system undergoes conformational change allowing the first proton transfer to QB- mediated via Ser264. After the second electron transfer to QBH system again adopts conformation allowing the second proton transfer to QBH-. The reduced QBH2 would then leave the binding pocket.
·Kulik N., Kuty M., Řeha D. The study of conformational changes in photosystem II during a charge separation. *J. Mol. Model.* (2020), doi: 10.1007/s00894-020-4332-9 (submitted 2019, accepted Feb 2020).

e) Another topic we are interested in and working on it is the development of enzyme catalysis in non-aqueous media in order to understand the technological applications of enzymes in non-conventional media such organic solvent, deep eutectic solvents, and ionic liquids.
Recently technological applications of biomolecules in non-aqueous such as organic solvents, ionic liquids, and deep eutectic solvents significantly expanded due to limitations of aqueous solutions. Non-aqueous media may enhance the solubility of hydrophobic substrates, prevent undesirable water-induced side reactions such as hydrolysis and alter the biomolecular activities. One of the main applications of organic solvent, ionic liquids, and deep eutectic solvents can be in bio-catalysis in non-aqueous media. Non-aqueous media due to their special physicochemical properties can be good potential to be used in enzyme catalysis, peptide synthesis, and DNA stabilization.
We have studied haloalkane dehalogenases enzymes in organic co-solvents such as acetone, formamide, and isopropanol. Molecular-dynamics simulations, time-resolved fluorescence spectroscopy, and steady-state kinetic measurements were employed to gain an insight into the mechanisms governing the enzyme–solvent interactions at the molecular level.
A broadly applicable computational method, involving molecular-dynamics simulations and quantitative analysis of co-solvent occupancies inside the access tunnels and active sites, was developed to aid the selection of an appropriate organic co-solvent. In this way, predicted changes in the accessibilities of the active site were confirmed by time-resolved and steady-state fluorescence spectroscopy and molecular dynamics simulations.
This work revealed that how organic solvents can influence the rate of enzymatic reactions. The outcome of this study has a very important impact on applications of enzymes on waste management and was published in the journal of CHEMBIOCHEM. This work was also breakthroughs in the field of enzyme engineering as it revealed the mechanism of activation of the enzyme in organic solvents.
As organic solvents have their disadvantage of volatility, therefore, replacement of them with a new category of solvents which are called ionic liquids can bring many advantages because many of them are not toxic and show almost no volatility, therefore, can be an excellent solvent for biochemical reactions.

Ionic liquids can be used in bio-catalysis as pure solvents, or co-solvents, in aqueous and biphasic systems as a replacement for conventional organic solvents where they have been used for lipases where interesting and promising results achieved.
The interesting aspect of ionic liquids is that they are salts constituted of an organic cation and/or anion where imidazole alkyl ammonium can be the cations with organic or inorganic anions. One of the best advantages of ionic liquids is the possibility of choosing an appropriate cation or anion to tune their physicochemical properties therefore they can be designed according to the need in different applications. By performing bio-catalysis in an appropriate ionic liquid many properties such as solubility of reagents and products, the stability of intermediate kinetics of the enzymatic reaction can be altered in desirable condition.
Moreover, recently we studied the activity of formate dehydrogenase from Pseudomonas sp. 101 in presence of different ionic liquids which revealed that some ionic liquids deactivate of the enzyme (due to penetration of some anions such as acetate to the active site), however other anions, such as CH3SO3-and BF4- do not affect the activity of the enzyme. Furthermore, in the low concentration of ionic liquids, the enzymatic activity was increased up to 42 % for mutated enzyme while no increase of activity was observed for the wild type enzyme.
As we have already studied the activity and stability of haloalkane dehalogenases and formate dehydrogenase enzymes in non-aqueous media therefor we want to extend our understanding of enzyme behavior in non-aqueous media. We started to study the recently discovered enzyme PETase from Ideonella sakaiensis strain 201-F6 bacteria which is capable of breaking Polyethylene terephthalate (PET) both experimentally and theoretically to find a solution for the problem of plastic materials in the environment. As the solubility of PET and other plastic materials in aqueous solution is very low and all enzymes including PET degrading enzyme perform degradation of plastic in aqueous solutions, therefore, the addition of co-solvent can affect both activity and reactivity of enzyme. In other words modification of aqueous media and application of co-solvent can be beneficial both for solubility of reactants (plastic materials), reactivity and stability of PET degrading enzyme.
One team members in Nove Hrady (B. Minofar) has been involved in understanding the structure and dynamics of hydrated non-aqueous media such as organic solvents, organic ions, ionic liquids and deep eutectic solvents in both bulk and surface by experimental and theoretical methods which are published in many international recognized journal such as journal of physical chemistry. Moreover, the bio-application of non-aqueous media for enzymes and other biomolecules has been published in peer-reviewed journals as bellow:
Aryafard, M., Abbasi, M., Řeha, D., Harifi-Mood, A. R., Minofar, B.; Experimental and theoretical investigation of solvatochromic properties and ion solvation structure in DESs of reline, glyceline, ethaline and their mixtures with PEG 400., J. Mol. Liq. (2019), 168..doi:10.1016/j.molliq.2019.03.149
Harifi-Mood A.R, Ghobadi R., Matić S, Minofar B., Řeha D., Solvation analysis of some Solvatochromic probes in binary mixtures of reline, ethaline, and glyceline with DMSO, Journal of Molecular Liquids, (222),2016, 845–853 http:// dx.doi.org/ 10.1016/j. molliq.2016.07.036
Zannotti, M.; Giovannetti, R.; Minofar, B.; Řeha, D.; Plačková, L.; D'Amato, C. A.; Rommozzi, E.; Dudko, H. V.; Kari, N.; Minicucci, M. Aggregation and Metal-Complexation Behaviour of THPP Porphyrin in Ethanol/water Solutions as Function of pH; Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 2018, 193.

D'Oronzo, E. , Secundo, F. , Minofar, B. , Kulik, N. , Pometun, A. A. and Tishkov, V. I., Activation/Inactivation Role of Ionic Liquids on Formate Dehydrogenase from Pseudomonas sp. 101 and Its Mutated Thermostable Form. ChemCatChem 2018, 10, 3247, doi:10.1002/cctc.201800145
Zeanat Zara, Saurabh K. Pandey, Babak Minofar, Ionic liquid based nano-materials for drug delivery. Springer Nature Singapore Pte Ltd. 2019, Online ISBN: 978-981-10-6739-6,

2. **Crystallogenesis and Biomolecular Crystallography** (assoc. Prof. Ivana Kuta Smatanova)

The main aim of the Kuta Smatanova's lab is in structural studies of the membrane and soluble biological macromolecular complexes using methods of X-ray diffraction. X-ray crystallography is the major technique to get the structure of biological macromolecules at atomic resolution. These protein structures are central to understand the detailed mechanisms of biological processes and to discover novel therapeutics using a structure-based approach. Several non-membrane membrane protein complexes have been crystallized in the lab.
The main projects in the evaluation period were focused on:

a) Structure-functional Relationships of Haloalkane Dehalogenases
This project is done in collaboration with Prof. J. Damborsky and Dr. R. Chaloupkova from Masaryk University Brno. Haloalkane dehalogenases (EC 3.8.1.5) are microbial enzymes with catalytic activity for the hydrolytic conversion of xenobiotic and toxic halogenated aliphatic compounds to corresponding alcohols. Structures of several novel enzymes crystallized are solved and deposited to Protein Data Bank within the framework of this project. Additionally, recently cloned and biochemically characterized enzymes are crystallized and structurally characterized. Although several molecular structures of haloalkane dehalogenases were known, some details of the reaction mechanism are not understood yet. Therefore, neutron diffraction is used in parallel to X-ray diffraction to describe the role of hydrogens in water and protonated residues in the active site and access tunnels connecting the active site cavity. Structural data is linked to the functional biochemical data leading to a better understanding of structure-function relationships of haloalkane dehalogenases and related alpha/beta-hydrolases.

·Liskova V., Bednar D., Prudnikova T., Rezacova P., Koudelakova T., Sebestova E., Smatanova I. K., Brezovsky J., Chaloupkova R. & Damborsky J. Balancing the Stability-Activity Trade-Off by Fine-Tuning Dehalogenase Access Tunnels. *Chemcatchem* (2015) **7**, 648-659, doi:10.1002/cctc.201402792.
·Tratsiak K., Prudnikova T., Drienovska I., Chrast L., Tratsiak K., Planas-Iglesias J., Daniel L., Prudnikova T., Brezovsky J., Bednar D., Smatanova I. K., Chaloupkova R. & Damborsky J. Deciphering the Structural Basis of High Thermostability of Dehalogenase from Psychrophilic Bacterium Marinobacter sp. ELB17. *Microorganisms* (2019) **7**, doi:10.3390/microorganisms7110498.
·J., Brynda J., Pachl P., Kuty M., Chaloupkova R., Rezacova P. & Smatanova I. K. Crystal structure of the cold-adapted haloalkane dehalogenase DpcA from Psychrobacter cryohalolentis K5. *Acta Crystallographica Section F-Structural Biology Communications* (2019) **75**, 324-331, doi:10.1107/s2053230x19002796.

·Havlickova P., Brinsa V., Brynda J., Pachl P., Prudnikova T., Mesters J. R., Kascakova B., Kuty M., Pusey M. L., Ng J. D., Rezacova P. & Smatanova I. K. A novel structurally characterized haloacid dehalogenase superfamily phosphatase from Thermococcus thioreducens with diverse substrate specificity. *Acta Crystallographica Section D-Structural Biology* (2019) **75**, 743-752, doi:10.1107/s2059798319009586.

b) RNA helicase P4 of bacteriophage φ4
This project is done in collaboration with Dr. R. Tuma from the University of South Bohemia in Ceske Budejovice. Bacteriophages package their genome into the empty capsid, which protects the genome from degradation from inside as well as outside of the cell. The genome packing is performed by molecular motor – P4 proteins, which translocates ssRNA into preformed empty viral capsids. The P4 poses an NTPase activity converting chemical energy from ATP hydrolysis to a mechanical movement of packing ssRNA precursors a procapsid, where replication and transcription of ds-RNA occurs. The P4s are RNA helicases belonging to the Superfamily 4 of helicases with the characteristic presence of conserved sequence motifs. The RNA helicases cause the distribution of RNA-protein complexes and carry out RNA unwinding. Crystallization and structure determination of P4 proteins from various bacteriophages helps to explain the exact function and mechanism the mechanism of molecular motors that converting chemical energy (usually from ATP hydrolysis) into mechanical work and translocate along nucleic acids, e.g. RNA and DNA helicases and chromatin remodelling proteins, many of which are associated with human diseases or viral infections and thus constitute potential drug targets. Detailed insight into the mechanism of ATP hydrolysis in P4 protein will help to design allosteric inhibitors for related ATP driven molecular machines.

The other projects we are working at are:
- “Structure-functional study of several Serpins from Ixodes ricinus” - in cooperation with Dr. J. Chmelar SBU Faculty of Sciences, Ceske Budejovice
- “BopN protein from Bordetella pertussis” - coordinated by Dr. L. Bumba from IMB ASCR in Prague
- “Crystallographic studies of bacteriophage lysins: Am24, Ap22, Ecd7, Si3 and St11 - in cooperation with Russian Academy of Sciences in Moscow
- “Structure-functional study of haloacid dehalogenases from Thermococcus thioreducens Tt80, Tt81 and Tt82” - in cooperation with Dr. M. Pusey and Prof. J. Ng from University of Alabama, Huntsville, USA

Advanced Optical Microscopy and cell biology (Dr. Josef Lazar)

The group of Advanced Optical Microscopy was focusing on technical improvements to the technique of two-photon polarization microscopy (2PPM) previously developed by the laboratory, and on developing new applications of this technique. We applied the technique to elucidation of the nature of interactions between G-proteins and G-protein coupled receptors in the resting state (A. Bondar, J. Lazar, J. Biol. Chem. 2017, fully authored by the team), as well as to interactions between potassium transporters and other proteins (A. Smidova & al., BBA-Mol. Cell. Research 2019, with the team contributing results of 2PPM observations). We have put considerable effort into developing the ability to use 2PPM for insights into the structure of

membrane proteins in living cells. Towards this goal, we verified the ability of the technique to determine molecular orientations with respect to lipid membranes using a simple synthetic model system (S. Timr & al., J. Phys. Chem. B, 2015; the team performed microscopy experiments, lead the effort and coordinated the work of others). In the following work, we developed a cellular system allowing making such determinations in proteins, and we demonstrated its usability (O. Rybakova & al., manuscript submitted, fully authored by the team). Finally, a major effort was invested in determining the directionality of the optical properties of fluorescent proteins. The results of this work are expected to find a wide range of uses by a large number of researchers. The uses will include interpretations of fluorescence energy transfer measurements, of polarization microscopy observations, but also of single-molecule observations. The work is highly multidisciplinary, novel, and required overcoming numerous challenges. We are currently preparing a manuscript describing the work and its results for submission to a prestigious scientific journal (the team playing a dominant role). The laboratory was involved in a collaborative project with teams from Ceske Budejovice and Austria, within which we implemented a stimulated Raman scattering (SRS) microscope. However, the system has been plagued by technical problems, both with the required lasers and with the microscope body/laser scanner.

Membrane Physiology and Bioenergetics (Dr. Jost Ludwig)

This research group has focused on functional analysis of ion channels and transporters, cation transport in yeast (Saccharomyces cerevisiae), cation homeostasis in yeast (more specifically: Localisation of cation transport proteins and regulatory proteins, cation flux measurements using ion-selective electrodes). The group is also active in the development of yeast and bacterial expression systems for ion channel genes, the development of Screening systems for compounds inhibiting transcriptional networks (MDR) and the development of production systems for pharmaceutically active peptides/proteins. Recent research was connected to SYSMO (Systems Biology on Microorganisms) within the EU-project TRANSLUCENT: Gene interaction networks and models of cation homeostasis in Saccharomyces cerevisiae. Structural and functional analysis of the yeast K+ translocation system(s) encoded by TRK1 and TRK2 Genes have been under investigation in Dr. Ludwig's laboratory. Here is some of some finding of Dr. Ludwig's laboratory.

Trk1 is the main K+-uptake system in S. cerevisiae. The protein consists of four domains (A-D) that are required to form a functional K+-selective pore on one polypeptide chain. Between domains A and B, Trk proteins possess a so-called "long hydrophilic loop" (LHL) comprising more than 50 % of Trk1's residues. LHL's function was unknown. By combining biochemical physiological and biophysical techniques (in vitro mutagenesis, generation of transgenic yeast, localisation of (mutated) GFP-tagged Trks via fluorescence microscopy and ion flux measurements using FLISE), a functional role could be directly assigned to LHL for the first time: its participation in the determination of ion selectivity and activity. By homology modelling, the results could be explained. The results were published in BBA-Biomembranes. The intellectual contribution of the team was >95 %. One experiment (determination of K and Na concentrations in yeast cells) was carried out in collaboration with the

University of South Bohemia in Ceske Budejovice, thus the contribution of the team in total work performed can be estimated as ~ 90-93 %.

A significant amount of work was dedicated to explore the “multimerity” of Trk systems. Trk cation translocation systems (and other proteins belonging to the “superfamily of K-transporters”, SKT) are related to K-channels. In contrast to most simple and ancestral K-channels whose principal ion translocating unit is made out of 4 identical or similar alpha subunits, each comprising one pore-forming domain (“MPM”), Trks possess 4 MPMs (A-D) on one polypeptide chain. Thus, Trks could be in principle functional as monomers. However, related proteins (KtrB and TrkH) were crystallised as dimers and for Trks, a tetramer has been proposed as the physiological unit. Using bimolecular fluorescence complementation (BiFC), it was shown that Trks are at least dimers. To distinguish between dimers and tetramers and to determine the interface region(s) between monomers, the team collaborated with lab 193 where models from Trk dimers and tetramers were generated in which the fluorescent protein fragments were inserted at different positions in Trk. The positions of the insertions allowed distinguishing between different di- and tetramers. The results showed that Trk1 is in principal a dimer with the interface made up of MPMs C and D. It seems possible though that when the genes are expressed at very low levels also monomers can exist and strong overexpression can lead to the clustering of Trks. To complement this data, single molecule fluorescence microscopy was performed in collaboration with FHOÖ Linz (Jaroslaw Jacak). A manuscript describing this data is in preparation. The contribution of CNSB to this work is ~90%, with 70% attributable to Lab 195 and 20% to Lab 193. Within the framework of elucidating structure and function of Trk systems, a study to characterize the not yet examined Trk from the biotechnologically important yeast Komagataella phaffii (formerly known as Pichia pastoris) was performed. PpTrk acts in a similar way as K+-uptake system as the homologous systems from S. cerevisiae but differs in ion selectivity. In collaboration with Lab 193, the probable structural basis for this difference was identified. A manuscript about this work is in preparation.

Ongoing cooperation between MBU-lab 195 and FHOÖ Linz, Austria, was established in which electrophysiological analyses of HUVEC cells are performed. The focus was on the analysis of cells grown on polymer structures generated in Linz by Nano-Lithography. This included measurements of K+ and Na+-fluxes in comparison to cells grown under standard conditions. Furthermore, the biocompatibility of polymers was tested by cell growth on these polymer structures and by analyzing the cell’s response to a Calcium signal (elicited by exposure to the calcium ionophore A23187). This included measurements of K+ currents via Ca2+ activated “MaxiBK”-channels. As controls, cells grown on clean coverslips were used. The results showed that cells grown on polymer structures do not differ in this respect from control cells grown on coverslips, confirming the inertness of polymers. The results of this project are supposed to be published this year.

In cooperation with Ruđer Bošković Institute in Zagreb, during this period, MIFE was used to measure transmembrane currents of cancer cells. In these experiments, electrophysiological reactions of various types of breast tissue tumor cells to application of a crown-ether compound, COM613, were measured. COM613 was

predicted to depolarize cell membranes and induce cancer cell apoptosis at higher concentrations. A manuscript including these results is in preparation.

Besides the mentioned specific activities, FLISE and MIFE are in constant development to increase their usability.