Final Report of the NKFIH K16 119516 project entitled "Exploring the first eukaryotic nicotinic acid catabolic pathway in the model organism *Aspergillus nidulans*"

The main focus of the project was the identification of the nicotinate catabolic pathway genes in *Aspergillus nidulans*, description of their regulation and deciphering the complete catabolic pathway (*Aim 1.*). We fulfilled all of these aims and published our results in three publications[1-3].

According to *Aim 1 sub-Aims 1.1.1., 1.1.2.,* we identified the nicotinate catabolic pathway-related genes and studied their regulation[1,3].
We found 11 co-regulated genes (*hxnS, T, R, P, Y, Z, X, W, V, M* and *N*) that are inducible by nicotinic acid and its downstream catabolic derivatives (6-hydroxynicotinic acid and 2,5-dihydroxypyridine), repressible by ammonium and they fall under the stringent control of the nitrogen-state sensitive GATA factor AreA and the specific transcription factor HxnR[1,3]. These genes are organized in three distinct clusters in *A. nidulans*, however this organization is variable across various species of the Ascomycota. We learned about cluster evolution by examining the cluster organization in Ascomycota, which revealed cases of gene duplication followed by or concurrent with integration in the cluster, partial or complete cluster loss, and horizontal gene transfer of a number of genes. These processes potentially reflect the plasticity and reticulate nature of the genesis of metabolic clusters[3]. **The *hxnR* gene was characterized by constructing both loss of function (including deletions) and constitutive mutants[1] and its putative DNA binding motif was also predicted.**
According to *Aim 1 sub-Aims 1.1.3., 1.1.4.* and *1.3.,* we generated 28 single, double and triple deletion strains both in *hxnR*$^{+}$ and constitutive *hxnR*^{c}*7* background by construction and transformation of gene-substitution cassettes into recipient strains or by using standard genetic crosses[2]. The resulting strains were tested for the utilization of the commercially available nicotinic acid derivatives as nitrogen-sources or as inducer precursors[2]. We also tested whether the accumulated intermediate compounds have toxic effect on the mutants. Finally, **the catabolism of 6-hydroxynicotinic acid in these strains was tracked by ultra-high-performance liquid chromatography – high-resolution mass spectrometry (UHPLC-HRMS) followed by the identification of the chemical structure of two purified, novel metabolites by NMR (Figure 1)[2]. The structural model of each enzymes was generated and compared to the structures of their closest structural homologs in order to further support the predicted functions of the nicotinate catabolism-related enzymes. All of the knowledge gleaned from our investigations was successfully used to construct the first complete nicotinic acid catabolic pathway involving all of the corresponding pathway-related enzymes and transporters and the intermediate compounds of each catabolic steps.**

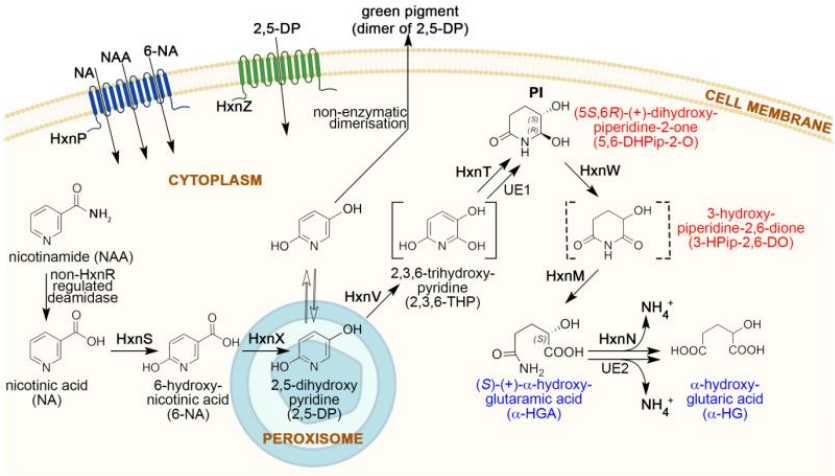

Figure 1. Nicotinate catabolic route in *A. nidulans*[2]. HxnP and HxnZ are transporters (represented by blue and green transmembrane domains, respectively) that transport the indicated compounds. HxnS hydroxylates nicotinic acid (NA) to 6-hydroxynicotinic acid (6-NA). HxnX operates in peroxisomes and converts 6-NA to 2,5-dihydroxypyridine (2,5-DP), which is subsequently hydroxylated by HxnV to 2,3,6-trihydroxypyridine (2,3,6-THP). HxnT and a yet-unknown alkene reductase (UE1) partially saturate the pyridine ring of 2,3,6-THP to (5*S*,6R)-(+)-dihydroxypiperidine-2-one (5,6-DHPip-2-O), which is then converted to 3-hydroxypiperidine-2,6-dione (3-HPip-2,6-DO) by HxnW, a NAD-dependent polyol dehydrogenase type enzyme. The ring of 3-HPip-2,6-DO is opened by the cyclic imidase HxnM between N-C2 resulting in (*S*)-(+)-α-hydroxyglutaramate (α-HGA) formation. The nitrogen is salvaged by HxnN amide hydrolase and results in α-hydroxyglutarate

(α-HG) formation. This reaction can also be catalyzed by other amide hydrolases (UE2). NA can be formed endogenously by the hydrolytic cleavage of amide group of nicotinamide (NAA) by a non-HxnR regulated deamidase. Cellular components as cell membrane, cytoplasm and peroxisome are shown and indicated by pictograms. Reaction in the peroxisome pictogram indicates the spatial separation of the referred catabolic step in the peroxisomes. The compound in square brackets denote a predicted intermediate that was not detected by UHPLC-HRMS method but deduced from the structure of the identified upstream and downstream metabolites. The structure of the compound in the dashed square brackets was deduced by the exact *m/z* value and MS/MS fragmentation pattern of the compound obtained by UHPLC-HRMS, the UHPLC-HRMS and NMR confirmed structures of the upstream and downstream metabolites in line with the proposed ketoreductase activity of the HxnW. UE: unidentified enzyme. PI: physiological metabolite inducer of the pathway related *hxn* genes; Compound names in red lettering denote metabolites, which have never been detected before neither in eukaryotic nor in prokaryotic organisms. Compound names in blue lettering denote metabolites not detected in prokaryotic NA catabolic pathways.

As we expected from the beginning of our studies, **the eukaryotic nicotinate catabolic pathway shows clear differences from previously described prokaryotic pathways**[2]. Steps of the saturation of the pyridine ring of 2,3,6-trihydroxypyridine (2,3,6-THP) to (5S,6R)-(+)-dihydroxypiperidine-2-one (5,6-DHPip-2-O) by the OYE-related alkene reductase HxnT (and a yet-unidentified enzyme) and oxidation of 5,6-DHPip-2-O to 3-hydroxypiperidine-2,6-dione (3-HPip-2,6-DO) by the ketoreductase/polyol dehydrogenase HxnW have hitherto only been detected in this pathway. Moreover, 5,6-DHPip-2-O is a completely new chemical compound. The ring opening of the piperidine ring occurs between C-N (by the cyclic imidase HxnM) generating (*S*)-(+)-α-hydroxyglutaramate (α-HGA), which has not been found previously in NA catabolic pathways. While no redundantly functioning enzymes are involved in the prokaryotic routes, two steps of the fungal catabolism involve alternative enzymes (two unidentified enzymes, one functioning redundantly with HxnT, the other with HxnN). **Catabolic steps downstream to 2,3,6-THP differ from those in prokaryotes and lead to the newly identified intermediate metabolites 5,6-DHPip-2-O and 3-HPip-2,6-DO.** The identification of these new metabolites may be of pharmaceutical or agricultural importance. In the near future we plan to screen the biological effect of 5,6-DHPip-2-O on various human cell lines and explore its applicability as a therapeutic agent or precursor.
The complete description of this eukaryotic pathway illustrated convergent evolution, both at the level of individual enzymes and at the level of a whole pathway.

In *Aim 1 sub-Aim 1.2.* we planned to investigate the subcellular localization of the pathway enzymes. N-terminal and C-terminal Gfp fusions of the pathway enzymes were generated and studied using fluorescent microscopy.
In the frame of this aim we showed **that HxnX is a peroxisomal enzyme**[2]. **We also found HxnW being localized to peroxisomes, however, no PTS signal is carried on this protein.** In order to determine whether the SYM motif at the C-terminal area of HxnW (15–18 AAs away from the C-terminal end) is in charge of peroxisomal transport, we investigated this SYM motif (recently described in plants as a PTS-1 signal). A Gfp tagged with the last 18 AAs from the C-terminal (Gfp-SYMTGQDIVVDGGVTRIS) failed to enter the peroxisomes, while a Gfp tagged with the SYM motif (Gfp-SYM) showed peroxisomal localization. With this study we did not clarified the reason of the peroxisomal localization of HxnW, however we revealed that **a C-terminal –SYM motif is a true peroxisomal PTS-1 signal for fungi**. We analyzed the occurrence of SYM motifs in the fungal kingdom (across the genomes of 993 fungal species) and characterized the function of those proteins that acquired C-terminal SYM. We found that SYM is a rarely occurring signal in the individual genomes and the overwhelming majority of the SYM-acquired proteins possess additional intracellular localization signals besidesSYM (mostly nuclear and mitochondrial localization signals) or they belong to the group of signal peptides (Figure 2).
We identified two proteins in *A. nidulans* with C-terminal SYM motif (AN1402 and AN5316). N-terminal Gfp fusions of these proteins showed that AN5316 localizes to the peroxisomes. AN1402 possesses nuclear localization signal too and the Gfp-AN1402 fusion protein dominantly was localized to the nuclei (the peroxisomal signal was rather weak). This work is used to prepare a manuscript entitled "C-terminal SYM motif is a rarely occurring fungal PTS-1 signal" by G. Varga, S. Nemuuzaya, S. Kocsubé, J. Ámon, C. Vágvölgyi and Z. Hamari. Funding by NKFIH OTKA is inidcated in the Acknowledgement paragraph.

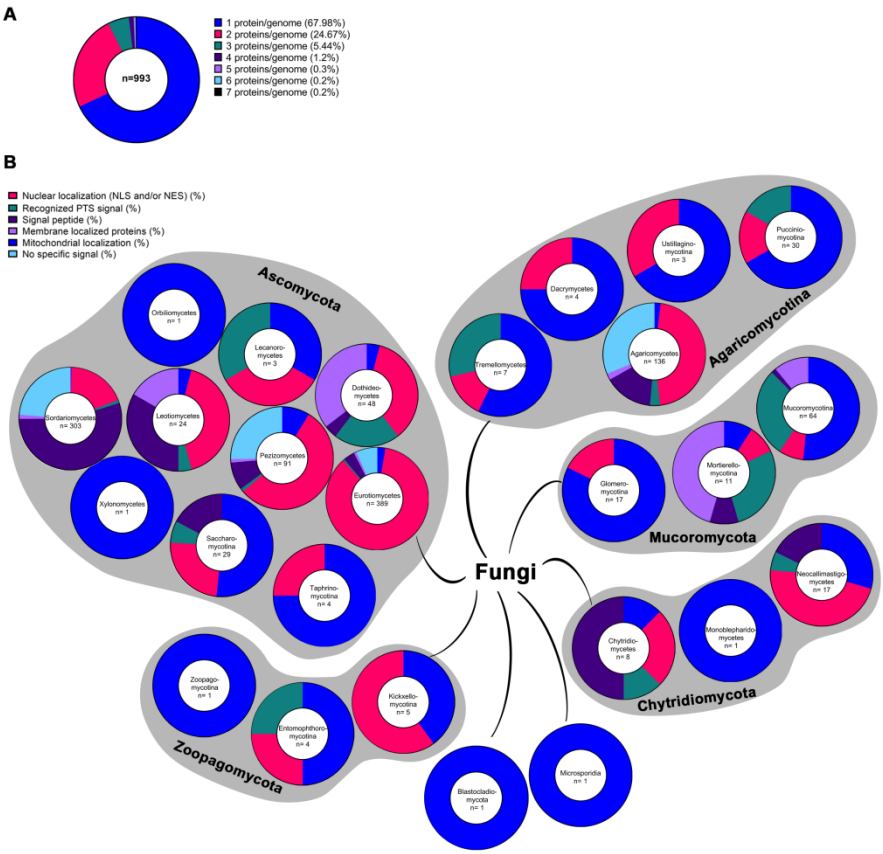

Figure 2. Occurrence of proteins with SYM terminal motif in fungal genomes and localization of these proteins according to their additional localization signals. (A) Frequency of the occurrence of C-terminal SYM motif in proteomes of individual fungal species (data were obtained by the analysis of proteomes of 993 fungal species on the bases of JGI Mycocosmos database). (B) Intracellular localization of SYM-acquired proteins within the major fungal taxons according to their additional localization signals (data based on the analysis of 1413 SYM-acquired proteins).

According to our hypothesis, proteins that typically are not localized in the peroxisomes under physiological settings but must be removed under adverse circumstances can be re-translocated into peroxisomes using the SYM peroxisomal signal. In the near future, by the study of the functioning of AN1402 we aim to investigate whether SYM-driven peroxisomal internalization can play a regulatory role for the SYM-carrying multilocalized proteins by modulating their spatial redistribution.

Since PTS signal was not identified on HxnW, we hypothesized that HxnW is co-transported to the peroxisomes by HxnX. The co-transportation mechanism is also known as piggyback transport. **By using a Bimolecular Fluorescence Complementation (BiFC) assay, we successfully revealed protein-protein interaction between HxnW and HxnX, which fully supports our hypothesis that HxnW enters the peroxisomes by piggybacking of HxnX. Additionally, we further supported this finding by showing that the Gfp-HxnW fusion protein fails to enter the peroxisomes in *hxnXΔ* background.** All of these experimental results are used to prepare a manuscript (entitled "The nicotinate catabolic-pathway-associated 5,6-dihydroxypiperidine-2-one ketoreductase HxnW enters the peroxisomes by piggybacking HxnX, an upstream enzyme of the pathway" by S. Nemuuzaya, G. Varga, E. Bokor, J. Ámon, C. Vágvölgyi and Z. Hamari), and will be submitted to in the near future. Funding by NKFIH OTKA is inidcated in the Acknowledgement paragraph.
We found that the rest of the pathway enzymes are localized in the cytoplasm.

Results of *Aim 1* were published in 3 research articles[1-3] and two manuscripts are prepared for submission (detailed above). The results were presented on international and national conferences in the form of posters or lectures (Conferences 1-14, see at the bottom), used to prepare two PhD thesis (PhD theses 1-2, see at the bottom) and 5 BSc/MSc theses (BSc and MSc theses 1-7, see at the bottom).

According to the original proposed plans, we initiated several nicotinate-pathway-related side-projects, which we pursued parallel with our main aim (*Aim 1*).

By working on the execution of *Aim 2. sub-Aim 2.1.*, we aimed to find out the mode-of-action of the pathway-specific transcription factor HxnR by studying the subcellular localization of the HxnR-Gfp and Gfp-HxnR fusion proteins under non-induced, induced and induced-repressed conditions. Despite we expressed the *hxnR-gfp* and *gfp-hxnR* fusion constructs not only from the physiological promoter of *hxnR* (P_{hxnR}) but also from constitutive (P_{gpdA}) and inducible (P_{prnA}) promoters, we could never fully complement the *hxnRΔ* phenotype. This strongly indicates that Gfp tagging of the transcription factor interferes with its functioning. Additionally, expression of the fusion constructs at the physiological level of *hxnR* never reached the fluorescent microscopy detection threshold level. We could only detect the fusion proteins when the fusion constructs were driven by P_{gpdA} or P_{prnA} promoters that resulted in fusion proteins above the physiological level. Under this circumstances, we could observe the formation of inclusion bodies in the nucleus, however we failed to see co-localization of HxnR-Gfp or Gfp-HxnR fusion proteins with the used chromatin marker, H1 histone. These negative results were presented on international and national conferences (Conferences 15-16, see at the bottom) and included in a PhD thesis (PhD theses 2, see at the bottom). At this point however, we terminated the further studies on this topic.

In *Aim 2. sub-Aim 2.2.* we attempted to decipher the evolution of the two paralogue enzymes, purine hydroxylase I (HxA) and purine hydroxylase II (HxnS) by swapping certain amino acid residues between HxA and HxnS, which we suppose to determine the substrate specificity.

HxA accepts only hypoxanthine and xanthine as substrates, while HxnS accepts only nicotinic acid and hypoxanthine as substrates. To reveal those amino acid changes, which had led to the neofunctionalization of HxnS during evolution, we generated, expressed and studied the activity of 13 mutated HxA- and 15 mutated HxnS enzymes for nicotinic acid, hypoxanthine and xanthine substrates in *hxnSΔ hxAΔ* and *xanAΔ hxAΔ* recipient backgrounds (Figure 3).We analysed the *in silico* 3D models of the modified proteins, tested the *in vitro* activity of these modified proteins by carrying out iodonitrotetrazolium chloride (INT) assays on native PAGE and tested the *in vivo* activity of these proteins too, by conducting growth tests on the modified protein expressing strains (the modified proteins were expressed constitutively in *hxnSΔ hxAΔ* and *hxAΔ xanAΔ* parental background). We found out that **HxA FTALF(1040-1044) and the corresponding HxnS region FATALH(1064-1069) are crucial for the hypoxanthine and nicotinic acid binding. Deletion of Ala1065 from the FATALH motif of HxnS (**with and without the additional change of the His residue to Phe) **results in gain of function for xanthine, reduction of function for hypoxanthine and loss of function for NA (Figure 3).**

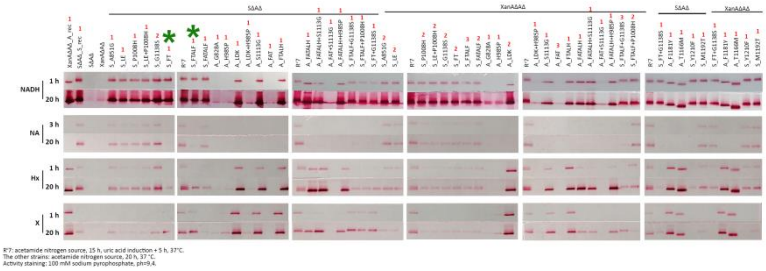

Figure 3. Iodonitrotetrazolium chloride (INT) assay with (HxA and HxnS are genuine NADH dehydrogenases), nicotinic acid (NA), hypoxanthine (Hx) and xanthine (X) as substrates. In the *hxnSΔ hxAΔ* background we detected strong xanthine activity, decreased hypoxanthine activity and lack of nicotinic acid activity in the FTALF (1064-1068) and FT (1064-1065) mutant HxnS expressing strains (lanes marked with green stars). The tetrazolium stained signals were documented after 1 h and 20 h of incubation as they are indicated on the figure. Recipient strain backgrounds are indicated above the lanes. Red numbers denote the copy numbers of the expression cassettes that were integrated in the genome.

In order to understand the structural basis of the activity-changes in the modified enzymes, we are currently conducting docking analysis in collaboration with a biochemist enzymologist expert, Cesar Millan from Universidad Autonoma del Estado de Morelos, Mexico. These analyses include the docking

of hypoxanthine, xanthine and NA substrates into the active center of the three dimensional structural models of the wild type and modified enzymes.
The *in vivo* tests carried out with the mutant proteins expressing strains together with the INT-NADH, INT-hypoxanthine, INT-xanthine and INT-nicotinic acid enzyme activity assays and with the structural models will be used to prepare a manuscript after the monitoring of more than one transformant strains expressing the mutated HxA and HxnS proteins.By interpreting the effect of the gain-of-function amino acid modifications at the structural level, we aim to identify the substrate binding residues and thereby better understand the structure and the function of xanthine dehydrogenases. We hope that this will help in the design and development of novel interacting substrates. The work on *Aim 2. sub-Aim 2.2.* were presented in one MSc thesis (MSc thesis number 8, see at the bottom) and two conferences (conferences 17 and 18, see at the bottom) so far.

In *Aim 3* we planned to produce and purify $(His)_6$-tagged Hxn enzymes with which we are able to conduct *in vitro* enzyme reactions, coupled with HPLC-detection to monitor the change of the supplied substrate concentration. We purified HxnX and HxnV, however the *in vitro* measurements had to be delayed due to the breakdown of UHPLC-HRMS machine for one year. This was the reason we asked the prolongation of the project execution time at February of 2020. After the machine was fixed, we fully focused on the metabolome measurements and analysis that served the identification of the metabolites of the nicotinate catabolic pathway. Due to shortage of time because of the priority of these measurements, we had to postpone the plans of *in vitro* enzyme characterization. In the near future, we shift our focus from HxnV and HxnX and plan to purify and characterize HxnT and HxnW, which we found out to be the most intriguing enzymes of the pathway. The product of HxnT and the substrate of HxnW, 5,6-DHPip-2-O, is a completely new metabolite and chemical compound, with potential pharmaceutical applicability. Therefore, the enzymes involved in the production or conversion of this compound bear with great practical importance.

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Conference presentations

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2. Eszter, Bokor ; Judit, Ámon ; Claudio, Scazzocchio ; Csaba, Vágvölgyi ; Zsuzsanna, Hamari. Structural homology function predictions for fungal nicotinate catabolising enzymes. In: 15th European Conference on Fungal Genetics (ECFG15): Program and Abstracts : Fungal genetics, host pathogen interaction and evolutionary ecology. Rome, Olaszország (2020) pp. 236-236
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PhD theses

1. Bokor Eszter, SZTE Biológia Doktori Iskola. "Az első eukarióta nikotinsav lebontási útvonal felderítése Aspergillus nidulans-ban" (2019) A védés időpontja: 2019-X-31 14:00, helye: SZTE TTIK Biológia Intézet, Ferenczy terem, Szeged, Közép fasor 52.

2. Ámon Judit, SZTE Biológia Doktori Iskola. "Az első eukarióta nikotinsav hasznosítási útvonal felderítése, szabályozása és enzimeinek vizsgálata Aspergillus nidulans modellszervezetben" (2018) A védés időpontja: 2019-II-18 13:00, helye: SZTE TTIK Újszegedi Biológiai Épület, Ferenczy Lajos terem, Szeged, Közép fasor 52.

BSc and MSc theses

1. Barsi Szilvia molekuláris bionika mérnöki BSc – 2016 Az HxnX fehérje intracelluláris lokalizációjának vizsgálata *Aspergillus nidulans*ban
2. Németh Dóra Tünde molekuláris bionika mérnöki BSc –Az HxnV fehérje intracelluláris lokalizációjának vizsgálata *Aspergillus nidulansban*
3. Birtyik Fanni biológia BSc 2018 - A *hxnNΔ* és *hxnVΔ hxnR^{c}7* törzs létrehozása *Aspergillus nidulans*-ban genetikai keresztezéssel és utódanalízissel
4. Eperjesi Beáta biológia BSc 2017 -Az *Aspergillus nidulans hxn* klaszter génjeire nézve dupla deléciós mutáns törzsek létrehozása
5. Ulbert Áron Balázs biológia BSc 2017 - Az purin hidroxiláz I enzimet kódoló hxA gén deléciója alX4 mutáns Aspergillus nidulans törzsben hxnR funkcióvesztéses mutánsok izolálása céljából
6. Szemerédi Nikoletta biológus MSc 2019 - A *hxnSΔ hxnTΔ*, illetve a *hxnSΔ hxnTΔ hxnYΔ* multideléciós mutánsok konstitutív *hxnR^{c}7* genetikai háttérbe helyezése *hxnR^{c}7* expresszáló integratív vektor transzformálásával
7. Omar Cisse Ochoa biológia BSc 2020 - Comparative study of 6-hidroxynicotinic acid utilization of *hxnS*Δ *hxnT*Δ*, cnxB11 hxnT*Δ and *hxB20 hxnT*Δ double mutant *Aspergillus nidulans* strains
8. Kanyó Andor biológus MSc 2021 - A purin hidroxiláz I és purin hidroxiláz II enzim aktív centrumában található aminosavak módosításának hatásai az enzimaktivitásra