Introduction

Although many microorganisms are able to utilize nicotinic acid (NA) as a sole nitrogen source, the degradation process was studied only in prokaryotes so far. The catabolic process is completely unknown in eukaryotes although it is known that some organisms, such as *A. nidulans* are able to utilize NA as the sole N-source. Very few data were available about the NA catabolism at the beginning of our research. The enzyme in the first step of the NA utilization (purine hydroxylase II/PHII - later HxnS) was characterized and mutants that cannot utilize NA were isolated. Interestingly, the identification of PHII was connected to the study of the purine utilization pathway. One of the key enzyme of the purine breakdown is the purine hydroxylase I (PHI, encoded by *hxA*) that can convert hypoxanthine (Hx) to xanthin and xanthine to uric acid. During the study of PHI functions the PHII enzyme was discovered. In PHI loss-of-function mutants Hx was successfully utilized in case the medium was supplemented with low amount (100 μM-1 mM) of NA or 6-hydroxynicotinic acid (6-NA). It was due to the production of PHII, which is able to convert Hx to xanthin, but is not able to convert xanthin to uric acid. The xanthin→uric acid transformation was found to be carried out by an alternative enzyme, an α-ketoglutarate-dependent xanthine dehidrogenase (XanA). The follow up studies revealed that besides Hx, PHII can also use NA as a substrate and can convert it to 6-NA. Several NA non-utilizer mutants were isolated in the 1970's and classified into linkage groups. Mutants of the *hxnS* group could not utilize NA, but could utilize 6-NA as sole N-source. Another group named *hxnR* were composed of mutants, that could utilize neither NA, nor 6-NA and could not grow on Hx media supplemented with the PHI inhibitor Allopurinol (Allp) and inducer amount of NA. They were thought to be regulatory mutants. Mutants of a third group named *aplA* could utilize NA and 6-NA more efficiently than the wild type strain and were able to grow on Hx media supplemented with Allp. Since NA induction was unnecessary in these strains to

perform PHII activity, *aplA* mutants were thought to be regulatory mutants. Other linkage groups were also defined in the proximity of the *hxnS*, *hxnR* and *aplA* linkage groups but those results were never published and the strains with one exception (*hxn6*) were lost with time.

In the 2000's our group started to reveal the first NA degradation pathway in the model organism *A. nidulans* by identifying the PHII encoding *hxnS* gene as AN9178 and the transcription factor coding gene *hxnR* as AN11197. We also revealed that *aplA* is the same locus as *hxnR*, and *aplA* mutations refer to gain-of-function mutations, which lead to constitutive HxnR. We studied the regulation of *hxnS* and *hxnR* together with their flanking genes (*hxnT, hxnY, hxnP* and *hxnZ*) and revealed a co-regulated cluster of six genes that are inducible by NA or 6-NA and their expression is depended on the transcription factor HxnR and transcription co-regulator AreA. We named this cluster NDC1. Through the obtaining of gene deletions for all *hxn* genes we studied their role in NA utilization. We concluded that *hxnT* and *hxnY* code for enzymes operating on an alternative route in the pathway, *hxnP* and *hxnZ* code for transporters and *hxnS* codes for PHII, which is involved not only in NA→6-NA conversion but in the further conversion of the 6-NA. We found out, that 2,5-dihydroxypyridine (2,5-DP) is an intermediate compound of the pathway and may serve as inducer. We showed that other genes outside of the cluster NDC1 are involved in the NA utilization.

Aims

The aims of the present study were the followings:

- Identification of *hxn6* mutation and other genes involved in the NA degradation pathway.
- Study of the regulation of the newly identified genes and the flanking genes by qRT-PCR if they are part of the degradation process.
- Search for and comparison of *hxn* gene orthologs by *in silico* analysis of the available fungal genomes in the database in order to reveal evolutionary relationships.
- Creating deletion mutants for the identified genes and examination of the NA utilization ability of the created mutants.
- Creation and study of multi-deletion mutants for *hxnS, hxnT* and *hxnY* genes.
- Identification of the cDNA sequences of the newly identified genes and their comparison with the database sequences.
- Investigation of the functions of the proteins encoded by the newly identified genes by *in silico* analysis.
- Study of the intracellular localization of the catabolic pathway by creating GFP-fused Hxn protein expressing strains and investigation these strains by fluorescent microscopy.
- Identification of the intermediate metabolites of the degradation process by GC-MS and HPLC-MS analyses with simple and multi-deletion mutants.

Methods

Microbiological methods

Cultivation of *E. coli* and *A. nidulans* strains on solid and in liquid media; growth tests.

Molecular methods:

DNA and RNA isolation from *A. nidulans*; cDNA synthesis; transformation of *A. nidulans*; competent *E. coli* preparation, chemical transformation of *E. coli*; plasmid isolation for *E. coli*; Southern blot analysis; PCR, qRT-PCR, Double-Joint PCR; DNA gel electrophoresis; molecular cloning; Sanger sequencing

Other methods:

Heterothallic crossing of *A. nidulans* strains; creation of GFP-fused constructions; fluorescent microscopy; *in silico* analyses; GC-MS and HPLC-MS analyses

Results

Identification of *hxn6* mutation and discovery of the NDC2 cluster genes

In order to find out what these unknown genes are, we started to examine the only extant mutant from the 1970's, *hxn6*, which was not able to utilize NA or 6-NA as a nitrogen source and was mapped outside of the NDC1 cluster, approximately 40 kb distance from the *hxnS* and *hxnR*. We knew that the identification of the *hxn6* locus would be very helpful for unravelling the genetic background of the NA catabolic pathway. By the transformation of the *hxn6* mutant with the plasmid gene bank of *A. nidulans* and selection for NA utilizing transformants followed by plasmid rescue and sequence analysis, we identified one suppressor gene of *hxn6*, AN11187. The AN11187 (later *hxnV*) is located approximately 40 kb from NDC1. The *hxn6* contains a G1171A nucleotide change that causes a W296STOP AA change in the protein resulting in chain termination. Subsequently we analysed the expression of the flanking genes of *hxnV* (AN9159, AN11172, AN9161, AN9162 and AN9163) by qRT-PCR in *hxnR*$^{+}$ and *hxnRΔ* strains under non-inductive and inductive (induced with 1 mM 6-NA) conditions. The *hxnV*, AN11172 and AN9161 showed co-regulation with the NDC1 genes (their expression depends on HxnR and NA derivatives) so these genes could be connected to the NA catabolic process. We named AN11172 *hxnW* and AN9161 *hxnX* and the gene cluster formed by them together with *hxnV* was named NDC2.

***In silico* homology search and discovery of NDC3 cluster genes**

In order to understand the evolution of the NA catabolic gene clusters we carried out an extensive *in silico* analysis of the HxnS, HxnR, HxnT, HxnY, HxnZ, HxnP, HxnX, HxnW and HxnV orthologs identified in nearly 200 *Pezizomycotina* genomes on JGI Fungal Genome Portal database using gene onthology BLAST search with synteny analysis. Remarkably, we found that the NDC1 and NDC2 genes are organized into a single cluster in most fungal species. We noticed the

conservation of certain gene pairs through the *Pezizomycotina*. *hxnS* was frequently coupled with *hxnT*, *hxnP* with *hxnY*, *hxnW* with AN6518 and *hxnV* with AN10833. Two of these genes, AN6518 and AN10833, were unknown for us. These genes are located next to each other on Chromosome I in the *A. nidulans* genome, which together with their conserved association with *hxn* genes through the *Pezizomycotina* raised the possibility of their role in NA utilization.

Transcript analysis of these two genes showed that they are co-regulated with the *hxn* genes, therefore AN6518 and AN10833 (under names *hxnM* and *hxnN*) can be regarded as new members of the NA utilization route, which form a third cluster, named NDC3.

cDNA sequencing

We constructed the protein models of the NDC2 and NDC3 genes through sequencing their cDNAs and found out that the protein model of *hxnV* in AspGD database is wrong.

***In silico* analysis of NDC2 and NDC3 proteins**

With the protein sequence of NDC2 and NDC3 genes we carried out *in silico* analysis, and hypothesized their possible function in the NA utilization. According to the *in silico* analysis:

- HxnV is probably a FAD-binding phenol monooxygenase and its substrate is supposedly 2,5-DP.
- HxnX has an N-terminal FAD-binding domain and it shows homology with monooxigenases. It may catalyze the hydroxylation of 6-NA to 2,5-DP.
- HxnW shows homology with members of the short-chain dehydrogenase/reductase (SDR) superfamily, oxidoreductases and NAD(P)-binding proteins. It is believed that it either acts as a ketoreductase or it

performs decarboxylation on an aromatic intermediate compound of the degradation pathway.

- HxnM is probably an amido-hydrolase which cleaves between a carbon and a nitrogen molecule in an intermediate of NA degradation pathway having a saturated pyridine ring.
- HxnN is an amidase, which supposedly cleaves the amide group from a compound with opened pyridine ring, that can be utilized as a nitrogen source thereafter.

Creating multi-deletion strains for *hxnS*, *hxnT* and *hxnY* genes

Based on our previous results we hypothesized that *hxnS*, *hxnT* and *hxnY* genes play role in the initial steps of the degradation pathway. In order to support this idea, we created *hxnShxnTΔ*, *hxnSΔ/hxnYΔ*, *hxnTΔ/hxnYΔ* and *hxnShxnTΔ/hxnYΔ* multi-deletion strains. Remarkably, *hxnShxnTΔ* and *hxnShxnTΔ/hxnYΔ* mutants showed more efficient 6-NA utilization properties than the wild type control. The explanation for this surprising phenomenon could be the lack of the shared promoter region of *hxnS* and *hxnT*, since we used a single substitution cassette for the creation of the *hxnShxnTΔ* double mutant in one step. In order to prove this theory, we created a double mutant, in which the deletion did not affect the promoter region. The new double mutant (with intact *hxnS/hxnT* promoter) showed the same improved 6-NA utilization phenotype as the old double mutants (with deleted *hxnS/hxnT* promoter). Therefore, the phenotype is independent from the presence or absence of the shared promoter region. To be able to explain this phenotype, further studies are required.

Creating deletion mutants for NDC2 and NDC3 cluster genes and examination of the created mutants

In order to study the function of the NDC2 and NDC3 gene products, we obtained gene deletion mutants for *hxnV*, *hxnW*, *hxnX*, *hxnM* and *hxnN* and tested their N-source utilization. Using Hx diagnostic media (Hx N-source supplemented with Allp and 100 μM NA or 6-NA), where only HxnS can catalyze the conversion of Hx to xanthine, we revealed that HxnM and HxnN operates downstream to the true metabolic inducer of the pathway (which is not NA or 6-NA) while HxnV and HxnX operates upstream to the true inducer. When we used NA or 6-NA as N-source *hxnVΔ*, *hxnXΔ* and *hxnMΔ* strains were not able to grow and *hxnWΔ* mutant together with *hxnNΔ* showed leaky phenotype. On one hand the result proved that the gene products of these genes play role in the NA utilization. On the other hand, the leaky phenotype of *hxnNΔ* foretells that HxnN is not the only amidase responsible for the cleavage of the amide group from the supposedly opened pyridine ring. The leaky phenotype of *hxnWΔ* on NA and 6-NA together with its successful growth on the diagnostic Hx+Allp+1 mM NA media indicated that HxnW operates on an alternative route upstream to HxnM and the true inducer metabolite.

Creating *hxnR^{c}7* deletion mutants for NDC2 and NDC3 genes and study of the created mutants

Since we don't know the true inducer compound (we know only that it derives from the metabolism of NA, 6-NA or 2,5-DP), we could not activate the gene expression of the *hxn* genes in those deletion mutants, which lack enzymes upstream to the true inducer. In order to circumvent the problem caused by the unknown true inducer, we introduced a constitutive *hxnR* allele (*hxnR^c*) into the *hxnVΔ*, *hxnXΔ*, *hxnWΔ*, *hxnMΔ* and *hxnNΔ* strains by genetic crossing. With the *hxnR^c* background the expression of the *hxn* genes is independent from the

production of the true inducer metabolite. As we expected, all these mutants were able to grow on Hx+Allp medium without NA-derived inducer. However, when the Hx diagnostic media was supplemented with 1 mM NA or 6-NA the *hxnVΔ hxnR*^{c} mutant could not grow at all and the *hxnWΔ hxnR*^{c} mutant showed a reduced growth. Interestingly, when the amount of inducer was decreased to 100 μM, both *hxnVΔ hxnR*^{c}*7* and *hxnWΔ hxnR*^{c} mutants could grow as much as the wild type. The explanation of this phenomenon is the toxic or HxnR inactivating nature of the compound accumulated in the constitutive *hxnVΔ* and *hxnWΔ* mutants.

We noticed that the *hxnVΔ hxnR*^{c} mutant produced a blue pigment on urea nitrogen source supplemented with 10 mM NA or 10 mM 6-NA. This phenomenon is similar to that seen in prokaryotes (*Bacillus spp.* and *Pseudomonas spp*.). The bacterial blue pigment is thought to be an azaquinone compound formed by a non-enzymatic conversion of an unsaturated trihydroxylated pyridine derivative. In order to identify and compare the fungal blue pigment to that of the prokaryotic, we are currently performing GC-MS analysis on samples derived from *A. nidulans hxnVΔ hxnR*^{c} mutant and *P. putida* NicX mutant.

To identify the enzymes which lead to the pigment production we created and tested *hxnYΔ/hxnVΔ hxnR*^{c}*7, hxnTΔ/hxnVΔ hxnR*^{c}*7, hxnWhxnVΔ hxnR*^{c}*7* and *hxnXhxnWhxnVΔ hxnR*^{c}*7* mutants for pigment production. Only the deletion of *hxnX* blocked the production of the pigment which proves our hypothesis that HxnX operates before HxnV in the pathway.

Investigation of the intracellular localization of HxnV and HxnX proteins

We aimed to study the intracellular compartmentalization of the NA catabolic route by expressing *gfp*-fused *hxn* genes in the appropriate *hxn* deletion strains. So far we studied the intracellular localization of HxnV and HxnX. According to the *in silico* localization signal search, HxnV was expected in the cytoplasm, while HxnX in the peroxisomes. In case of the HxnV-GFP we could

not exclude the possibility of compartmentalization. Further studies on the HxnV-GFP expressing strain is needed. In case of the GFP-HxnX we clearly proved the peroxisomal localization of the fused protein, by showing the co-localization of GFP-HxnX with PTS-tagged mRFP.

GC-MS and HPLC-MS analyses with simple and multi-deletion mutants

In order to unravel the chemical structure of the pathway related compounds, in collaboration with a chemist expert Dr. Mónika Varga (operating at the analytical work station of the Department of Microbiology at the USz) we performed GC-MS and HPLC-MS analyses on samples obtained from cultures of constitutive single and multiple-deletion mutants incubated with NA or 6-NA substrates after pregrowth. The multiple deletion mutants were created by genetic crosses (*hxnMΔ/hxnXΔ*, *hxnMΔ/hxnVΔ*, *hxnMΔ/hxnWΔ*).

Although the preliminary GC-MS and HPLC-MS experiments has already provided valuable data for the uncovering of certain features of the NA catabolic pathway, the identification of each step of the pathway remains a challenging and robust work done in the near future.

The primary component analysis of the metabolites obtained from the *hxnXΔ, hxnWΔ, hxnVΔ* and *hxnMΔ* simple and the *hxnMΔ/hxnXΔ, hxnMΔ/hxnVΔ* és *hxnMΔ hxnWΔ* double deletion mutants confirmed that the HxnV, HxnW and HxnX enzymes operate in the pathway upstream to HxnM in two alternative routes. The investigation of *hxnSΔ, hxnTΔ* and *hxnYΔ* simple and their multiple deletion mutants supported that the NA degradation pathway of *A. nidulans* splits to alternative routes. Based on the clusterization of the primary components of the *hxnSΔ, hxnTΔ* and *hxnYΔ* simple and their multiple deletion mutants we can propose that HxnS operates upstream to HxnY and HxnT operates downstream to HxnY on a route, which is alternative to that involving HxnW. We observed that most of the metabolites accumulated in more than one mutants, which indicates the

possibility of reversing the pathway steps. Only 2,5-DP and an unidentified compound with 131 g/mol molecule weight (Mw131) could be found exclusively in single mutants. The 2,5-DP was exclusively found in the *hxnVΔ* strain, that clearly suggests that the 2,5-DP is the substrate of HxnV. The Mw131 compound was exclusively found in the *hxnWΔ* strain, which indicates that Mw131 is the substrate of HxnW. We proposed that Mw131 is a 1,2,3,6-tetrahydropyridine-2,3,6-triol, which is a more saturated derivative of the intermediate 2,3,6-trihydroxypyridine compound of the prokaryotic routes (in *Bacillus* and *Pseudomonas spp*.). Remarkably we observed NA accumulation in *hxnVΔ, hxnWΔ* and *hxnXΔ* samples from experiments, where the substrate treatment was carried out with 6-NA. The only explanation of this phenomenon is the back-conversion of 6-NA to NA. This result contributed to the understanding of the improved 6-NA utilization of *hxnSΔ/hxnTΔ* double deletion mutant (detailed above). Since the back-conversion of 6-NA to NA was detected in many mutants, we propose that other metabolites downstream to 6-NA can be reversed. Probably the different kinetics of the reverse reactions can explain why we found NA accumulation only in 3 mutants.

Summary

During this project:

- we identified the mutation in the *hxn6* strain and discovered the *hxnV, hxnW* and *hxnX* genes, which together form the NDC2 cluster
- we analysed nearly 200 *Pezizomycotina* fungi genome for *hxn* orthologs and discovered *hxnM* and *hxnN* genes, which formed NDC3
- by cDNA sequencing we found out that the sequence of *hxnV* is not correct in the AspGD database
- we created many single and multiple-deletion mutants in order to collect more details about the NA catabolic pathway:

- *hxnSΔ::pabaA*$^{+}$, *hxnSΔ/hxnTΔ, hxnSΔ/hxnYΔ, hxnTΔ/hxnYΔ, hxnSΔ/hxnTΔ/hxnYΔ*
- *hxnVΔ, hxnXΔ, hxnWΔ, hxnMΔ, hxnNΔ*
- *hxnMΔ/hxnVΔ, hxnMΔ/hxnXΔ, hxnMΔ/hxnWΔ*
- *hxnYΔ/hxnVΔ*, *hxnTΔ/hxnVΔ*, *hxnWhxnVΔ, hxnXhxnWhxnVΔ*

- we investigated the NA utilization ability of the created mutants and we concluded that:
  - *hxnV, hxnW, hxnX, hxnM* genes are involved in the steps of the pathway after 6-NA
  - *hxnV* and *hxnX* genes play role in the degradation before the true inducer of the pathway is created
  - *hxnW* is probably involved in an alternative pathway
- we created HxnV-GFP and GFP-HxnX expressing strains and by using fluorescent microscopy we found out that HxnV is probably localized in the citoplasm, while HxnX is localized in the peroxisomes
- by HPLC-MS analyses we found out that the substrate of *hxnV* is 2,5-DP, the substrate of *hxnW* is 1,2,3,6-tetrahydropyridine-2,3,6-triol, and that there is a back-conversion of certain intermediate compounds in the degradation process

Our results significantly contributed to the understanding of the NA catabolic pathway, and proved that the eukaryotic route is different from those found in prokaryotes in many aspects. They are the split of the pathway to alternative routes and production of intermediate compounds that were not detected in the prokaryotic routes. Despite of our achievements, further research is needed for the complete understanding of each steps of the pathway.

Publications

Publications in referred journals summarizing the results of this Ph.D. Thesis

Bokor, Eszter; Ámon, Judit; Keisham, Kabichandra; Karácsony, Zoltán; Vágvölgyi, Csaba; Hamari, Zsuzsanna; HMGB proteins are required for sexual development in *Aspergillus nidulans*; PLOS ONE 14 : 4 p. e0216094 (2019) ; **IF: 1,95**

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Cumulative impact factor: 6,798

Conference abstracts summarizing the results of this Ph.D. thesis:

Bokor E, Ámon J, Vágvölgyi Cs, Hamari Zs; Investigation of the initial steps of the nicotinic acid degradation pathway in *Aspergillus nidulans*; In: [Department of Public Health Faculty of Medicine University of Szeged] 19th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and abstracts. 65 p. Szeged, Hungary, 09.06.2017-10.06.2017 Szeged: University of Szeged, Faculty of Medicine, 2017. p. 15. (ISBN:978-963-306-535-8)

Bokor Eszter; Investigation of the nicotinic acid degradation pathway in *Aspergillus nidulans*; Acta Biologica Szegediensis 60:(1) p. 80. (2016) Conference for Doctoral Students in Biology. Szeged, Hungary: 17.05.2016 -18.05.2016

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