Final Report - OTKA NNF 78714
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The project reulted 1 scientific pbublication and 2 conference presentations, where the reference number of the contracted project was cited:

Hamari Z., Amillis S., Drevet C., Apostolaki A., Vágvölgyi C., Diallinas G., Scazzocchio C. (2009) Convergent evolution and orphan genes in the Fur4p-like family and characterization of a general nucleoside transporter in *Aspergillus nidulans*. Mol. Microbiol. 73(1)**:** 43-57.

Hamari, Z., Vágvölgyi, C., Scazzocchio, C. (2009) Identification of FUR-4-like nucleobase transporter family in *Aspergillus nidulans*. 11th regional conference on environment and health. Abstract. 15/16 May 2009, Szeged, Hungary

Z. Karácsony, Z. Hamari (2010) Construction of a bidirectional *E. coli*/*Aspergillus nidulans* autonomous replicative expression vector. Annual Conference of MMT (Hungarian Society of Microbiologists), Keszthely, Hungary 13-15/October/2010. Mycology Section poster: MP-1.

1. Mutant gene banks from human hCNT1 cDNA and hCNT1 cDNA derived non-functional E497Q-hCNT1 molecule (for technical control purpose; E497Q-hCNT1 construction is described in section 2) were constructed and used for screening of gain-of-function mutants.

The primary goal was to develop a mosaic patterned partial mutant gene bank from human hCNT1 (O00337) cDNA. According to the suggestions of the reviewers the experimental approach to select small parts of the cDNA molecule for intensive mutagenesis had been changed for *in silico* analysis approach. Altogether 14 sections of hCNT1 cDNA were selected for mutagenesis. The flanking sequences were to be left native. First we tested the critical technical process, the development of the mosaic patterned molecule. Primers for the PCR amplification of 2x14 cDNA sections were designed to be overlapped with the neighbouring primer by 10 nucleotides. Assembly of the complete cDNA molecule by Stemmer's *in vitro* recombination process (Stemmer 1994 PNAS 91:10747-10751) from their 28 sections has been attempted several times without success. Besides the 1:1 molecule ratio of the purified template molecules, several ratios different from 1:1 were repeated without success. Further we focused on the development of the mutant gene bank by a more conventional approach. Here the entire cDNA molecule was subjected to random mutagenesis through error-prone PCR, and then the mutations were combined by subsequent repetition of Stemmer's *in vitro* reassembly process. Besides the hCNT1 cDNA molecule we also carried out the procedure on a non-functional mutant molecule, E497Q-hCNT1 cDNA (construction is described at section 2) for the purpose of using the mutant gene bank of E497Q-hCNT1 cDNA for the technical evaluation of the carried out process. Due to a random mutagenesis a backmutation event is expected on the non-functional E497Q-hCNT1 molecule that results in the formation of a functional molecule that could be isolated

when the E497Q-hCNT1 mutant gene bank is transformed into *cntA::riboB pyrG89 pantoB100 riboB2 pyrG89 A. nidulans* strain. During the development of hCNT1 and E497Q-hCNT1 mutant gene banks error-prone PCR was carried out by five consecutive repetitions where products of a previous PCR were used as template in the following reaction. The series of error prone PCRs were followed by three consecutive repetitions of Stemmer's *in vitro* molecule disassembly/assembly procedure that resulted the random combination of the primary mutations. Mutant gene banks were finally constructed by the transformation of the mutated molecule populations of hCNT1 and E497Q-hCNT1 into pNNPanto expression vector (development of the vector is described in section 4). The quality of the mutant gene banks were monitored by restriction analysis of 250 independent clones. The quality of the hCNT1 mutant gene bank was assessed to be satisfactory. Two transformations and direct selections were carried out with *A. nidulans* strains *uapA24 uapC201/401 azgA4 pantoB100* (uptake deficient for hypoxanthine and uric acid) and *furA::riboB pantoB100 yA2 riboB2* (uptake deficient for allantoin) (development of strains are described in section 6) without resulting a gain-of-function mutant. The mutant gene bank of E497Q-hCNT1 was transformed into *cntA::riboB pyrG89 pantoB100 riboB2* strain and 2 transformants were isolated on uridine supplemented minimal media. The appearance of two transformants clearly indicated that the process of mutant gene bank construction was technically correct. Probably the gene bank was not rich in mutants. Regardless, it is worth to repeat the transformation processes in case of hCNT1 mutant gene bank. In any case, the existing hCNT1 mutant molecule population can be subjected to further series of mutagenesis processes and a more extensively mutated gene bank can be developed for the isolation of gain-of-function mutants.

2. A non-functional E497Q-hCNT1 mutant hCNT1 cDNA molecule was constructed by directed point mutagenesis for the purpose of generation and using the E497Q-hCNT1 mutant gene bank to evaluate technically the process of mutant gene bank generation.

The main purpose of generating a point-mutant non-functional hCNT1 molecule was to track the steps of mutant gene bank construction and in case of failure of the application of hCNT1 mutant gene bank, the technical failures can be detected or excluded. The G (Guanine) in position of 1489 in the cDNA had been changed to C (Cytosine) by PCR based directed mutagenesis that resulted the change of glutamate (E) to glutamine (Q) at the residue 497 in the hCNT1 molecule. The generated E497Q-hCNT1 molecule is expected to fail to be expressed at the surface of the cell membrane that results uridine uptake deficiency. The E497Q-hCNT1 molecule was transformed into *cntA::riboB pyrG89 pantoB100 riboB2 pyrG89 A. nidulans* (description of mutant construction is described in section 3) and transformants were not detected. To complement the *pyrG89* mutation the *cntA::riboB pyrG89 pantoB100 riboB2 pyrG89* strain needs to take up extracellular uridine by an active transporter. When the E497Q-hCNT1 is subjected to random mutagenesis, and a mutant gene bank is generated, then it

is expected to detect back-mutants where the native nucleotide is reestablished at position 1489 that results a functional uridine carrier transporter. A back mutant can be isolated by transformation of *cntA::riboB pyrG89 pantoB100 riboB2 pyrG89* strain and by selection on uridine supplemented minimal media.

3. *cntA::riboB pyrG89 pantoB100 riboB2 A. nidulans* deletion mutant was developed by crossing *cntA::riboB pyrG89 pyroA4 biA1 riboB2* with *pantoB100 riboB2* for the purpose of test-transformation of (i) hCNT1 and E497Q-hCNT1 in the heterologue expression system; (ii) transformation with E497Q-hCNT1 mutant gene bank.

The *cntA::riboB pyrG89 pantoB100 riboB2 A. nidulans* deletion mutant was developed by crossing *cntA::riboB pyrG89 pyroA4 biA1 riboB2* (Hamari et al. 2009 Mol. Microbiol. 73: 43-57) strain with *pantoB100 riboB2* mutant. The developed *cntA::riboB pyrG89 pantoB100 riboB2* strain meets the demand of testing the heterologue expression of hCNT1 and E497Q-hCNT1 cloned into pNNPanto expression vector (vector construction and test transformation are described in section 4 and 5, respectively); and the requirements needed for transformation with E497Q-hCNT1 mutant gene bank. This strain is not able to grow on uridine and pantothenic acid supplemented media unless a functional uridine transporter is expressed the strain. (To complement the *pyrG89* mutation the *cntA::riboB pyrG89 pantoB100 riboB2 pyrG89* strain needs to take up extracellular uridine by an active transporter.)

4. Novel autonomous expression vector of *A. nidulans*, pNNPanto was developed by engineering the existing pAnGFP integrative GFP fusion *A. nidulans* vector in four steps.

The integrative *E. coli* based *A. nidulans* expression vector pAnGFP (Pokorska et al. 2000 J.Mol.Biol.298:585-596) was reengineered to achieve an autonomous replicative, pantoB markered expression vector that support the generation of mutant gene banks and the transformation of suitable *A. nidulans* recipient strains (see sections 3 and 6). The following reengineering processes were carried out on the original pAnGFP vector: the *amaI* replication origo sequence was inserted, *pantoB* selection marker sequence was inserted, GFP coding region was eliminated and the flanking *Pgpd* constitutive promoter and *trpC* termination seqences were kept and *Nde*I/*Not*I cloning sites were built in between them; the single native *Nde*I site of the original vector had been disrupted by cloning the *pantoB* marker sequence onto blunt-ended *Nde*I site. The constructed pNNPanto expression vector was 13.2 kb in size (Karácsony and Hamari 2010, poster presentation at the annual conference of Hungarian Society of Microbiologists at 2010 October, Keszthely, Hungary).

5. Heterologous expression of the human hCNT1 and E497Q-hCNT1 and the expression of *A. nidulans* CntA (AN5493.3) cDNAs cloned in pNNPanto autonomous expression vector were carried out in *cntA::riboB pyrG89 pantoB100 riboB2* recipient strain.

The *cntA::riboB pyrG89 pantoB100 riboB2* strain is not able to produce uridine endogenously due to the pyrG89 mutation and deficient in extracellularly available uridine uptake due to the deletion of the gene for the executive uridine transporter, *cnt*. When hCNT1 cDNA cloned in pNNPanto vector had been transformed into this recipient

strain, transformant colonies could be isolated. By plasmid rescue process it was demonstrated that all of the transformants carried the hCNT1 expression vector and the hCNT1 produced a functionally active transporter. By transformation of the same recipient strain with E497Q-hCNT1 cDNA cloned in pNNPanto we confirmed that the E497Q-hCNT1 mutant molecule is deficient in the uptake of uridine. The expression vector itself was tested and evaluated by cloning and expressing the native uridine transporter of *A. nidulans* (CNT) in the recipient *cntA::riboB pyrG89 pantoB100 riboB2* strain.

6. Hypoxanthine/uric acid and allantoin uptake deficient mutants were developed for the purpose of direct screening for gain-of-function mutants.

For the screening of hypoxanthine/uric acid gain-of-function mutants *uapA24 uapC201/401 azgA4 pantoB100* strain was developed by cross of *uapA24 uapC201/401 azgA4 pabaA1* strain with *pantoB100 riboB2* mutant.

For the screening of allantoin gain-of-function mutants the *furA::riboB pantoB100 yA2 riboB2* strain was available as a result of our previous work on transporters (Hamari et al. 2009 Mol. Microbiol. 73**:** 43-57).