**MOLECULAR MICROBIOLOGY 2024**

***CONTENT OF LABORATORY EXERCISES***

***50% final grade***

**Exercise 1**

**1.1 Mutations - Phenotypes in *Aspergillus nidulans* : an excellent genetic model system for basic research**

**Exercise 2**

**2.1 Genetic crossing of strains of Aspergillus nidulans-**

***Step I: generation of stable heterokaryon***

**2.2 Recording the results of Exercise 1**

**Exercise 3**

**3.1 Genetic crossing analysis - *Step II: isolation of cleistothecia and incubation of ascospores***

**3.2 Mutagenesis in *Aspergillu*s *nidulans* via the “*Minos”* transposable element**

**Exercise 4**

**4.1 Cell Microbiology - Study of protein trafficking dynamics by fluorescence microscopy in living cells (in vivo) -**

***Monitoring the subcellular topology of the UapA transporter physiological and mutant forms***

**4.2 Recording of genetic crossing analysis - progeny phenotypes**

**4.3 Recording of mutagenesis via the *Minos* transposable element**

**Exercise 5 (homework)**

**Protein sequence *decoding* and other *in silico* structural analyses**

**Basic principles of phenotype analysis in model fungi**

**Introduction: *Aspergillus nidulans* as a model genetic eukaryotic system**

*Aspergillus nidulans* is a simple eukaryotic organism. It is a non-pathogenic filamentous fungus, easy to use genetic, biochemical and molecular techniques. Essential genetic analyses (both phylogenetic and non-phylogenetic) have been developed since 1950. Genetic transformation, specific gene replacement, plasmid integration and rescuing, and studies of the kinetics of transmembrane transporters of solutes were developed in the 1980s and 1990s. Nucleotide sequencing of *the A. nidulans* genome (2.7x107 bp, approximately 9,500 genes) has been completed since 2006 which allowed the identification of homologous genes with known functions of scientific interest. Like all soil fungi, *A. nidulans* has an excellent metabolic repertoire. It grows in the presence of a wide variety of nutrients which it can use as sources of nitrogen and/or carbon.

The life cycle, which can be asexual, sexual or paraphyletic (parasexual), offers excellent opportunities for genetic and developmental analysis (Figure 1). In the asexual cycle of *A. nidulans*, a haploid conidiospore swells and buds, a germ tube elongates to produce the first mycelial segment (known as *germlings*), within which the conidiospore nucleus moves and divides mitotically to eventually produce the multinucleate mycelium. The mycelium is a network of connected segments (hyphae), each segment containing multiple nuclei (*coenocytic*). The mycelium, in turn, differentiates into aerial hyphae and *conidiophores*, where *conidiospore* chains are produced form specialized cells called *metulae* and *phialidiae* (see inset on the right). Conidiospores are characterized as inert and have very low levels of metabolism.

***Εικόνα 1: Aspergillus nidulans asexual and sexual life cycles***

The dormant phase of conidiospores is interrupted when the fungal environment is hydrated and acquires properties ideal for further growth when there is a source of nitrogen and carbon, resulting in rapid swelling, redefinition of the organization of the nucleus, the appearance of the early hyphae and finally the formation of the mature mycelium. This process of conidiospore germination can be divided into three stages: the isotropic growth phase, in which hydration of the spores is sufficient, the polarity establishment phase and the polarity maintenance phase.

The isotropic phase of growth involves spore swelling, uncoiling of the chromatin of the nucleus, and a series of changes in the surfactant properties at the periphery of the cell, reflected by an increased tendency for spores to adhere to wet culture. In the primary stages of this phase (20 min), in the presence of water alone, a number of metabolic pathways, such as respiration, are developmentally activated, and much later (2-4 h) basic protein and nucleic acid biosynthesis and extracellular enzyme production will follow. Trehalose hydrolysis plays a key role in the activation of conidiospores through its osmoregulatory role. Of particular interest is that during isotropic growth phase of A. nidulans, regardless of what the growth medium contains, the expression of plasma membrane transporters is activated for the direct uptake of substances/metabolites/nutrients such as nucleotide bases, amino acids, nitrate and ammonium ions, etc. The expression of these transporters is null in the dormant spore phase, while in the fully developed mycelium phase it is maintained at low levels, unless transport substrates are present in the nutrient (induction effect). The expression of the transporters is followed by the expression of the relevant enzymes for the ingested substances.

During the sexual cycle of *A. nidulans*, which usually occurs under stress conditions, a pair of nuclei divides in a synchronous manner. Initially two haploid mycelial nuclei are fused and the resulting diploid nucleus undergoes reduction, followed by two mitoses, leading to the formation of eight haploid ascospores within an ascus. In the process of nuclei division, extensive genomic recombination can occur. Ascus formation is carried out clonally to eventually produce a fruiting body, the cleistothecium, within which hundreds of ascospores (~104 ascospores) are enclosed. The ascospores are released by rupturing the cleistothecium. The sexual cycle in a single strain is regarded as a *selfing* phenomenon.

When two different strains come into contact, the fused textures (anastomosis) form a heterokaryon. The heterokaryon is not stable, but under stress conditions, e.g. when we have different fluctuations for each strain, a balanced ratio of two different nuclei and maintenance of the heterokaryon is possible. If a breeding cycle in the heterokaryon follows, new strains will arise due to genetic recombination. The phylogenetic cycle that takes place in a heterokaryon through the fusion of different parental nuclei is considered a phenomenon of genetic *crossing.*

In ***A. nidulans*** there are hundreds of available strains with mutations in genes involved in biosynthetic and catabolic pathways. Strains that are unable to biosynthesize specific metabolites, such as vitamins, amino acids and nucleotide bases (fluxes), serve as 'host' plasmid strains in genetic transformation experiments. In these experiments, plasmids carry as gene markers for selection of transformed strains the genes that are "absent" in the recipient strains (e.g. *argB-* strain is transformed with plasmids carrying *argB+* to select only the transformed strains in the absence of arginine in the growth medium - see course notes). Most transformation plasmids in *A. nidulans* are *integration* plasmids (homologous or heterologous, single or multiple; see course notes). Plasmids integrated into the A*. nidulans* genome can also be 'rescued' either by PCR or by transformation in *E. coli.* In the latter case, the rescue process is based on the ability of *A. nidulans* to excise plasmids integrated into its genome during mitosis. The elimination of the plasmid is the result of homologous recombination between two consecutive copies of the gene of interest, sequentially integrated into the fungal genome, leading to the elimination of one of the two copies carried on the plasmid.

**For more information on *A. nidulans* see e-class articles 1-5**

[**https://eclass.uoa.gr/modules/document/?course=BIOL331**](https://eclass.uoa.gr/modules/document/?course=BIOL331)

**EXPERIMENTAL PART OF EXERCISE 1**

***1.1 Mutations & phenotypes in Aspergillus nidulans: An excellent genetic tool for basic research***

*Purpose of the exercise*. A great advantage of *A. nidulans* (and other ascomycetes) is that mutations are reflected in many different colony phenotypes growing on different growth media. With this exercise we will look at phenotypes of catabolism, anabolism or growth (morphology changes) and related mutations, and discuss whether a mutation appears to be a *total* or *partial* loss-of-function or a *modification* of an existing function (these concepts will also be discussed in class).

***A. nidulans* phenotypes related to:**

**Use of C & N sources**

Nutrients: a) MMG+ammonium, b) MMG+UA, c) MMF+ammonium

*A. nidulans* strains: *pabaA1* (wt), *Δ3 pabaA1*, *Δ7 pabaA1*

Per laboratory bench of 6, each group (2 persons) inoculates 1 dish of a-c with all three strains.

MMG =minimum nutrient with glucose

MMF: minimum nutrient with fructose

UA: uric acid,

Δ3 & Δ7 = strains with 3 or 7 inactivated purine transporters and related metabolites\* (to be discussed in the exercise)

**Auxotrophies**

Nutrients: a) MMG/NH4+ + riboflavin, paba, b) MMG/NH4+ + riboflavin, pantothenate, c) MMG/NH4+ + pantothenate, paba

*A. nidulans strains*: *pabaA1, riboB2, pantoB100*

Per laboratory bench of 6, each group (2 persons) inoculates 1 dish of a-c with all three strains.

**Morphological Variations & Temperature Influence**

Nutrients: CM (complete nutrient)

Mutant strains\* of *A. nidulans*: *Δap2, hulAΔC2, sedVts, wt*

Per laboratory bench of 6, each group (2 persons) inoculates 1 dish with all three strains. The three groups on the bench are each incubated at a different temperature (25, 37, 42 o C)

*\*We will discuss what the relevant mutations are*

**EXPERIMENTAL PART OF EXERCISE 2**

***2.1 Genetic crossing of Aspergillus nidulans***

**Step I: generation of heterokaryon**

We will cross *A. nidulans* strains by inoculation in CM, 1 cm apart, of the two parental strains with complementary auxotrophies necessary for selection of crossed clostridia later. You will transfer the site where heterokaryotic hyphae have been generated, to a new Petri dish without any of the auxotrophic supplements necessary for parental growth and survival. This is how you will select/enrich the heterokaryon. At the same time, we will "deprive" the heterokaryon of air (cover with transparent membrane) to create stress, which *induces* sexual differentiation, fusion of 2 nuclei, and the development of 8 *ascospores* through meiosis and 2 mitoses in *asci*, which are enclosed in developing cleistothecia (visible black spheres on the mycelium) . This takes 14 days.

You will receive *A. nidulans* crosses:

*yA2 riboB2 x wA pantoB10 uaY462C*

*Δ3 pabaA1 x pantoB100 yA2*

Per lab bench of 6, each group (2 individuals) will transfer, as shown, the fusion region of the hyphae to an MMG/ON3 dish with 1 of the 2 crosses.

***2.2 Recording - photography - analysis of the results of exercise 1***

**EXPERIMENTAL PART OF EXERCISE 3**

***3.1 Genetic crossover analysis***

**Step II: Isolation of cleistothecium and isolation of ascospores**

Each group (2 persons) isolates a single cleistothecium and analyses the progeny of the cross as we will be shown to you.

For each section:

12 MMS dishes for clamp cleaning

12 MMG/NH4 tubes for finding crossed cleistothecia

***3.2 Mutagenesis in Aspergillus nidulans via the Minos transposon***

You will receive 1 Petri dish per group of the ***Minos/Tpase*** strain *UapA-GFP ΔalX pyroA4/pabaA1*

Isolate the conidiospores with a spatula and prepare their suspension under completely sterile conditions

Inoculate MMG/allantoin tryptic through nutrient top-agar to select for uric-acid resistance mutants. The dishes will be incubated for 7 days at 37 °C.

*Instructions for the above will be given during the exercise.*

The mechanism of the Minos/Tpase transposon will be described in the class/course and in article

Minos as a novel Tc1/mariner-type transposable element for functional genomic analysis in Aspergillus nidulans. Evangelinos M, Anagnostopoulos G, Karvela-Kalogeraki I, Stathopoulou PM, Scazzocchio C, Diallinas G. Fungal Genet Biol. 2015 Aug;81:1-11. doi: 10.1016/j.fgb.2015.05.007. Epub 2015 May 25.

**EXERCISE 4 - INTRODUCTION**

**Cell Microbiology - Study of protein trafficking dynamics by In vivo Fluorescence Microscopy**

**Monitoring the subcellular topology of the UapA transporter (normal and mutant forms)**

Membrane protein biogenesis includes a) ER-exit, b) vesicular trafficking via Golgi and endomembranes, c) targeting to plasma membrane or other membranes, d) endocytosis e) turnover by sorting in the vacuole or by autophagy, f) recycling to the PM. Very little is however known about the biogenesis and degradation of transport proteins (*transporters*), which is the most abundant type of membrane proteins in all eukaryotic cells. This is mainly due to the difficulty in identifying and isolating them, due to the polytopic and hydrophobic nature of the transporters and their location on the membrane. In addition, the very low expression levels of these proteins, combined with their relatively low abundance in the membrane (often < 0.01%), makes it difficult to apply classical immunofluorescence and immunoprecipitation methods. Most immunolocalisation studies have been described on yeast membrane proteins and are based on the overexpression of the membrane transporter of interest.

The development of ***green fluorescent protein*** (**GFP**) technology (and other fluorescent proteins, RFP, YFP, BFP, etc.) has proven to be extremely beneficial for the study of cellular protein expression, and for the identification of *cis* and *trans elements or factors* involved in the processes of membrane protein traffic and dynamic topogenesis. In addition to its often higher sensitivity compared to immunolocalization, GFP technology has the outstanding advantage of *in vivo* localization.

GFP has been widely used to study the expression, subcellular trafficking, topogenesis and regulated degradation of transmembrane transporters of *A. nidulans*. As a reference gene for subcellular localization of transmembrane transporters in living fungal cells *in vivo*, we use an activated version of the *gfp* gene (sgfp), which was shown to be particularly functional in *A. nidulans*. In all cases, chimeric transporter-GFP proteins are generated by standard genetic engineering, and are expressed through the either *endogenous* or a *strong* and *regulatable* promoter.

The open reading frame of GFP is almost always fused to the carboxyl-terminal end of each transporter gene in such a way that the chimeric transporter-GFP protein is translated as a single transporter (in the absence of a stop codon). It has been found that the length of the transporter-GFP amino acid linkers in the chimeric protein is often important for the functional expression and topogenesis of chimeric proteins in the membrane and for GFP fluorescence. Chimeric molecules with 0, 2 or 4 amino acid linkers are often functional. The chimeric transporter-gfp gene is incorporated into the normal genetic locus by homologous recombination, following genetic transformation of *A. nidulans* with linear DNA "cassettes", or via ectopically integrated genome plasmids carrying the chimeric gene (see lesson), always in a strain whose "relevant" endogenous transporters are inactivated (why?)

Chimeric transporter-GFP expression allows the study not only of wild-type transporters, but also of a multitude of available *mutant forms*, resulting in the identification and classification of mutations affecting subcellular trafficking, stability, targeting and degradation of transporters, from those that exclusively affect the activity and kinetics of the transporters *per se.*

Read e-class (articles 8-12):

* Dimou S, Diallinas G. Life and Death of Fungal Transporters under the Challenge of Polarity. Int J Mol Sci. 2020 Jul 29;21(15):5376. :
* Diallinas G, Martzoukou O. Transporter membrane traffic and function: lessons from a mould. FEBS J. 2019 Dec;286(24):4861-4875.
* Dimou S, Martzoukou O, Dionysopoulou M, Bouris V, Amillis S, Diallinas G. Translocation of nutrient transporters to cell membrane via Golgi bypass in Aspergillus nidulans. EMBO Rep. 2020 Jul 3;21(7):e49929.
* Martzoukou O, Karachaliou M, Yalelis V, Leung J, Byrne B, Amillis S, Diallinas G. Oligomerization of the UapA Purine Transporter Is Critical for ER- Exit, Plasma Membrane Localization and Turnover. J Mol Biol. 2015 Aug 14;427(16):2679-96.
* Martzoukou O, Diallinas G, Amillis S. Secretory Vesicle Polar Sorting, Endosome Recycling and Cytoskeleton Organization Require the AP-1 Complex in Aspergillus nidulans. Genetics. 2018 Aug;209(4):1121-1138.

**EXPERIMENTAL PART OF THE EXERCISE 4**

We will study under fluorescence microscope natural strains & and mutant strains expressing the **UapA** (urate and xanthine uptake) transporter of *A. nidulans*. You will get cultures in dishes suitable for observation in an inverted epifluorescence microscope, from:

Groups 1-6 UapA-GFP in nitrate, ammonia or uric acid

Groups 7-9 UapA-Y47A-GFP in nitrate

Groups 10-12 UapA-GFP on thiAp-sec24 strains in nitrate

Microscopy will be performed on the microscope located in the Physiology Department in groups of 8, 1 group per 40 minutes.

Comment on the subcellular location of the **UapA** you have observed (keep images of all your results) in each case, and based on what will be said in the exercise and lectures, you will give a written report in the form of a scientific report with images and commentary (1 page).

**4.2 Recording of genetic crossing analysis - progeny phenotypes**

Comment on your result.

**4.3 Recording of mutagenesis via the Minos transposable element**

Make a note of the procedure you will do in the presence of me and the exercise assistants. Based on your results and what will be said in the exercise and in the lesson, develop discussion, hypotheses and conclusions as to the nature of possible mutations that might arise (see lesson)

**EXERCISE 5**

**Decoding of protein sequences in silico**

We will become familiar with:

* Protein analysis: Primary protein structure-analysis, functional patterns, patterns of post-translational modifications, etc. Secondary protein structure-analysis (α-helices, β-folds, etc.), transmembrane regions, structural patterns. Tertiary protein structure-homology threading
* Comparison with other sequences in databases (blastp), multiple sequence alignments, phylogenetic analyses, etc.
* Genome annotation
* Protein mining and associated information

***Some essential URLs for in silico analysis of proteins***

• http://www.ncbi.nlm.nih.gov/PubMed/

• http://www.expasy.ch/

• http://web.expasy.org/translate

• https://www.predictprotein.org/

• http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html

• http://embnet.vital-it.ch/software/TMPRED\_form.html

• http://www.cbs.dtu.dk/services/TMHMM/

• http://www.psort.org/psortb/index.html

• https://toolkit.tuebingen.mpg.de/

• <http://www.phylogeny.fr/>

* <http://tcdb.org/>
* http://blanco.biomol.uci.edu/mpstruc/

***Genome annotations link***

* http://www.yeastgenome.org/
* http://www.aspgd.org/
* http://www.fgsc.net/
* http://genome.jgi.doe.gov/programs/fungi/index.jsf
* <http://fungidb.org/fungidb/> (if available this year)
* <https://genome.jgi.doe.gov/programs/fungi/index.jsf>

**EXPERIMENTAL PART OF EXERCISE 5 (for home)**

- What interesting information can you find *in silico* by annotating the **Scs2** protein from the S*. cerevisiae* genome database (Yeast DB) or Uniprot?

-Find the possible transmembrane regions of the **DfnA** protein of *A. nidulans*. Which of them are amphiphilic (α-helical wheel projection)?

- Find 20 fish proteins that show significant similarity to the **UapA** protein of *A. nidulans*. Compare the sequences (multilalign & esprit). Do you observe conserved patterns? Comment.

- Create a phylogenetic tree (phylogeny.fr or other program) of **Sec13** homologs with representatives from all major fungal groups. Find conserved patterns and annotate.