

### An alternative strategy for cloning minor permeases in *Aspergillus nidulans*

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The *uapC* gene was cloned by complementation of the cryosensitive phenotype of *uapA* mutants following the "instant gene bank" method. The analysis of the transformants is presented and a strategy to clone other transport related genes is proposed.

In the filamentous fungus *A. nidulans*, several permeases responsible for the uptake of different nitrogen and/or carbon sources have been identified. According to early genetic evidence, the *uapA* gene codes for a major permease involved in the uptake of uric acid (UA), xanthine, their thioanalogues 2-thiouric acid and 2-thioxanthine (2TX), allopurinol and oxypurinol (Darlington and Scazzocchio 1967, J.Bacteriol. 93: 937-940). This gene has been cloned, its sequence determined and its expression studied at the level of message accumulation by Northern blot experiments. It has been shown that *uapA* expression is highly regulated, induced by uric acid and repressed by ammonia (Diallinas and Scazzocchio 1989, Genetics, 122:341-350, Gorfinkiel *et al.*, 1993 J.Biol.Chem., 268:23376-23381). Loss of function mutations in *uapA* result in a leaky phenotype, characterized by reduced but nevertheless substantial growth on uric acid and xanthine as nitrogen source at 37°C. However, when plates are incubated at 25°C growth of *uapA* mutants is drastically reduced. This suggests that either the other permease(s) involved in uric acid-xanthine uptake or additional mechanisms of active or passive transport are not functional at the lower temperature. In fact, UA transport mediated by UapC, the general purine transporter of *A. nidulans* is not functional at 25°C (Diallinas, Gorfinkiel and Scazzocchio, unpublished observations).

In order to identify genes whose products could complement the cryosensitivity of *uapA* mutants we made use of the "instant gene bank" method for gene cloning by mutant complementation in *Aspergillus nidulans*. This method uses ligation *in vivo* of donor DNA and an autonomously replicating plasmid (Gems *et al.*, 1994 Mol. Gen. Genet. 242:467-471). A strain carrying a deletion of the *uapA* gene -*ΔuapA*- and the mutation *argB2* is unable to grow on UA or xanthine as sole nitrogen source at 25°C and is auxotrophic for arginine. This strain was transformed with total genomic DNA isolated from the *ΔuapA* strain, purified by CsCl density gradient ultracentrifugation and partially digested by *Sau3A* restriction enzyme to obtain an average fragment length of 4-6 kb. The cloning vector pDHG25 (construction described in Gems *et al.*, 1991 Gene 98: 61-67), contained the AMA1 element, promoting autonomous replication in *Aspergillus*, and the *argB* gene for initial selection of transformants. The vector was made linear at its unique *Bam*HI site. Three micrograms of the vector was mixed with 10 μg of the donor DNA and used in transformation of about 5 X 10<sup>7</sup> protoplasts of *A. nidulans* strain *argB2 ΔuapA*. The transformation mixtures were plated in standard Petri dishes with osmotically buffered protoplast regeneration minimal medium supplemented with UA and incubated at 25°C. Ten thousand *ΔuapAargB<sup>+</sup>* transformants were scored and one colony growing on the selective agar plates at the restrictive temperature was obtained after 5 days of incubation. The purified transformant grew on minimal medium containing UA as sole nitrogen source better than the recipient strain and showed an irregular growth phenotype (Fig. 1). Plasmid rescue in *E. coli* was performed as described previously. Twenty ampicillin resistant colonies were tested and all of them contained the same plasmid of 21-22 Kb, pG1. This plasmid is capable of re-transforming the *ΔuapAargB2* strain to arginine prototrophy/growth in UA at 25°C at an efficiency of 30 colonies per μg of DNA. These "second generation" transformants grew better than the initial one in plates with UA and were more sensitive to the effect of 2-TX. (Fig. 1)

The stability of the transformants was examined by plating conidia on non-selective medium and subsequent replica plating onto arginine-free minimal media with UA as sole nitrogen source at the restrictive temperature. The original transformant showed a 17% of mitotic instability while the second generation transformants lost their plasmid in 55-60% of the asexual progeny.

The rescued plasmid contained the *uapC* gene as evidenced by its restriction pattern enzyme analysis (Fig. 2). Strongest evidence was provided by PCR amplifications. The rescued plasmid and a plasmid containing the previously cloned gene were amplified by PCR with two different pairs of *uapC* specific oligonucleotides. The same band is amplified in both plasmids (Fig. 3). Moreover, the PCR products obtained with oligos 10 and 12 (Fig. 3, lanes 3 and 4) were precipitated and cut with *Ava* I restriction enzyme. The expected fragments of 490 and 101 bp were obtained, showing the identity of the amplified DNA and the *uapC* sequence. The PCR products obtained with oligos 17 and 20 (Fig. 3, lanes 8 and 9) were precipitated and digested with *Hpa* I. The expected fragments of 318 and 293 bp were obtained showing again the identity of the rescued plasmid insert and the *uapC* gene (Fig. 4).

This gene had already been cloned by heterologous hybridization with a *uapA* specific probe. It codes for a broad specificity, low affinity permease able to incorporate all natural purines and their thioanalogues in the cell (Diallinas *et al.*, 1995 J.Biol.Chem. 270:8610-8622).

Southern blot analysis of genomic digested and undigested DNA from the original and secondary transformants (not shown) suggested the presence of multiple copies of the cloned gene in the latter.

This strongly suggests that while a single copy of the *uapC* gene (the resident endogenous copy) does not allow growth of a *uapA* deleted strain on UA as sole nitrogen source at 25°C, multiple copies do. Alternatively, as already described, an ectopic integration of *uapC* gene may lead to a non cryosensitive transport activity.

The results presented here describe the cloning of the *uapC* gene by complementation of a cryosensitive phenotype. Both the recipient strain and the donor DNA were wild type for this gene. The methodology employed can be used to clone other minor permeases or transport related genes for which there is no mutant known. To avoid the cloning of *uapC*, the donor strain should therefore contain a deletion of this gene.

The UapC residual activity is not detected under a given assay condition whereas the presence of multiple copies of the gene responsible for it bypass the restrictive condition. Any gene responsible for a residual activity, not detected under a given condition (low temperature in this case) can be cloned in this way provided the presence of multiple copies of the gene responsible for it bypass the restrictive condition.

Moreover, this procedure can be extended to other pathways or functions to clone genes responsible for residual activities detected only upon the complete loss of function mutations in the main gene.

This work was partially supported by IFS.

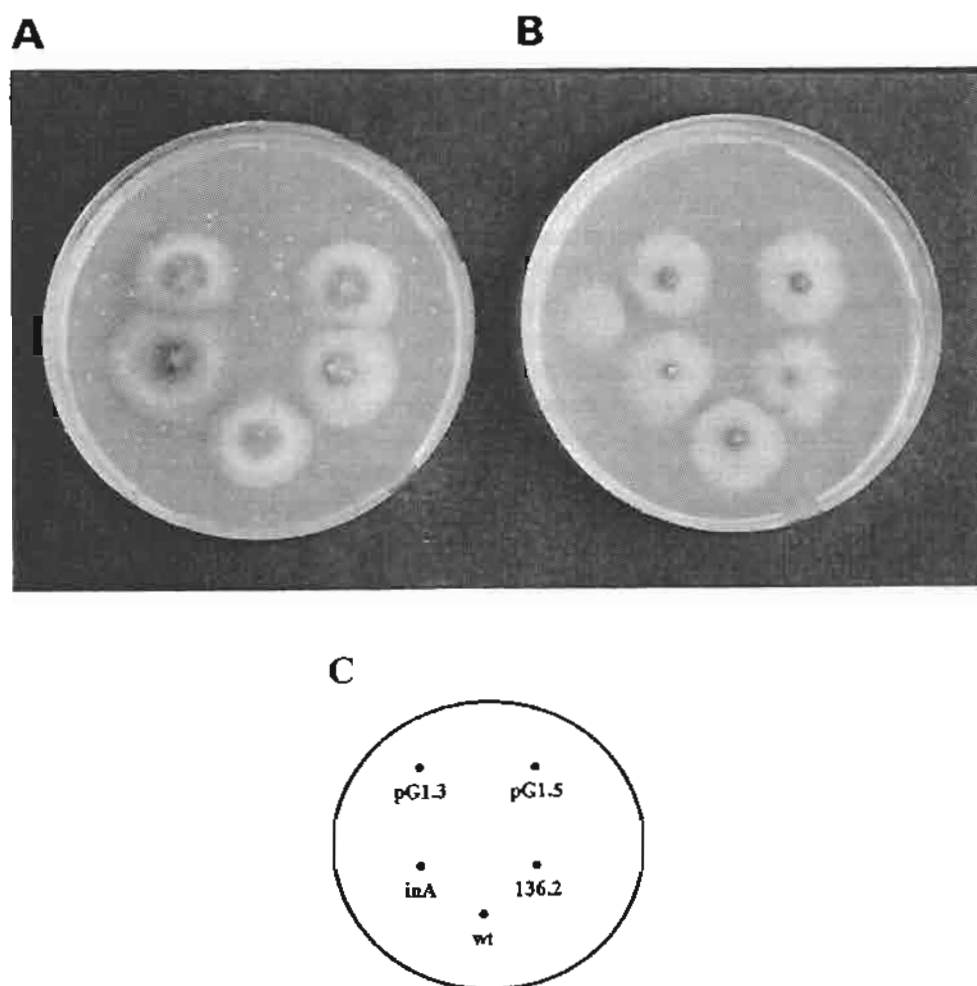


Figure 1. Growth tests of the transformants. Growth was monitored on appropriately supplemented minimal medium with 1% (w/v) D-glucose as carbon source and A) 3.3 mM of ammonium (as the (+)-tartrate) as nitrogen source in presence of 0.1 mg/ml of 2-thioxanthine and B) 0.1 mg/ml of uric acid as nitrogen source after 5 days at 25°C. C) Strains: wt: wild type for purine permease, inA: *uapA* inactivated, 136.2: original transformant, pG1.3 and pG1.5: second generation transformants (*ΔuapAargB2* strain transformed with pG1).

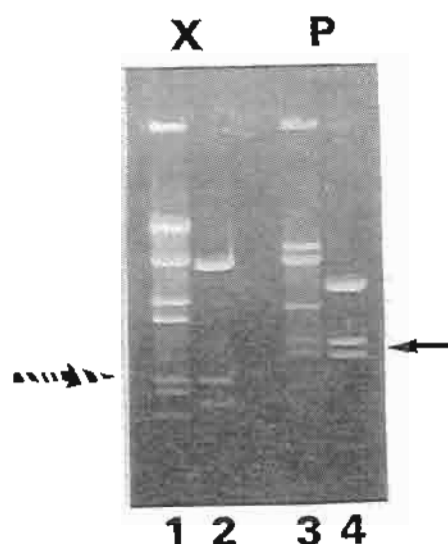


Figure 2: *Xho*I (lanes 1,2) and *Pst*I (lanes 3, 4) digestions of pG1 (lanes 1 and 3) and pAN903 (a plasmid containing the *uapC* gene cloned, lanes 2 and 4). The 1.5 and 1.6 kb bands of the *Pst*I digestion (black arrow), and the 0.9 kb band of the *Xho*I digestion (hatched arrow) are characteristic of the restriction enzyme pattern of the DNA sequence of the *uapC* gene.

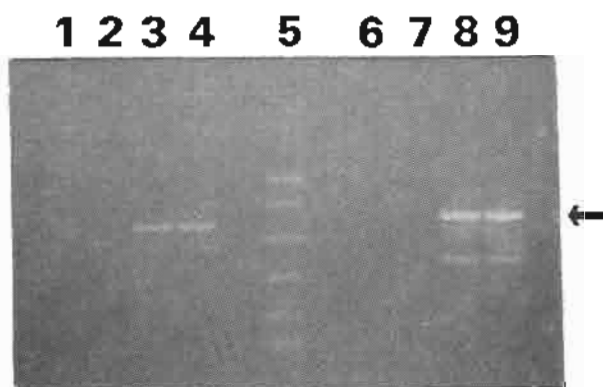


Figure 3: Products of PCR amplifications performed with *uapC* specific oligonucleotides. Lanes 1 to 4: oligo 10 (5' ATTCAGGGTGGCGTCC 3') and 12 (5' GACCAGAGGAACCAGGC 3') were used. Lanes 6 to 9: oligo 17 (5' GGCAACGCAATGAAAGAC 3') and 20 (5' CCATTACAACGAGGAAGAAGC 3') were used. Lanes 1 and 6: no DNA was added to the reaction, lanes 2 and 7: pDHG25 was used as DNA template, lanes 3 and 8: pAN903 -the plasmid containing the previously cloned gene- was used as DNA template, lanes 4 and 9: pG1 -the rescued plasmid- was used as DNA template. About 10 ng of each plasmid were amplified by 35 cycles under the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 56°C and elongation for 1 min at 74°C using the *Taq* polymerase (Boehringer, Mannheim). The expected products of 591bp (oligos 10 and 12) and 611 bp (oligos 17 and 20) were obtained and run in a 1.5% agarose gel. Lane 5: PCR markers (Promega) was used, sizes of the fragments: 50, 150, 300, 500, 750 and 1000 bp. The *uapC* sequence had been previously submitted to the GenBank™ with accession number X79796.

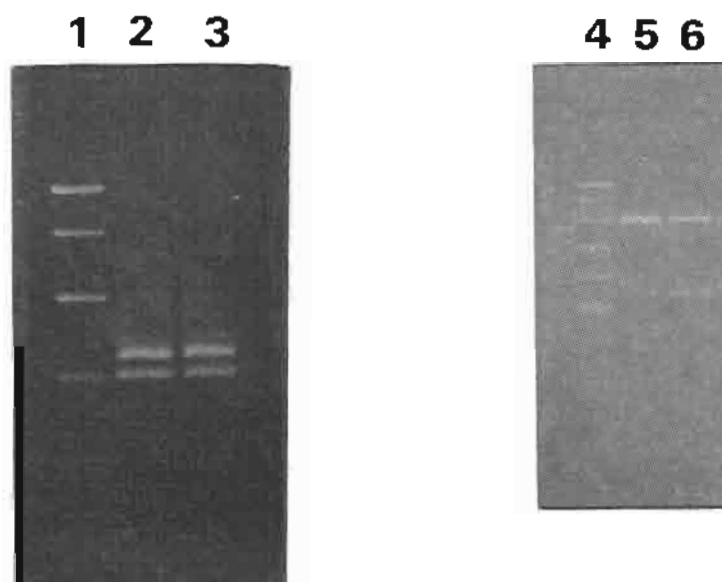


Figure 4: Restriction enzyme digestion of the PCR products. Lanes 2 and 3: Hpa I digestion of the 611 bp DNA fragment amplified with oligos 17 and 20. The expected fragments of 318 and 293 bp are obtained. Lanes 5 and 6: Ava I digestion of the 591 bp DNA fragment amplified with oligos 10 and 12. The expected fragments of 490 and 101 pb can be observed. Lanes 1 and 4: PCR marker.