How to use the *mtr* gene as a selectable marker for loss or gain of function

Luis M. Corrochano

Background

The product of the *mtr* gene is an amino acid permease required for the uptake of neutral aliphatic, and aromatic amino acids, like tryptophan (Koo and Stuart 1991, Dillon and Stadler 1994). In addition, the MTR permease allows the entry of toxic amino acid analogs, like *p*-fluorophenylalanine (FPA) or 4-methyltryptrophan, in the cell. The name of the locus, *mtr*, stands for methyltryptophan resistance. The ability of the MTR permease to allow the entry of amino acids or toxic compounds facilitates the selection for loss or gain of *mtr* function. A double mutant strain *mtr trp-2* will grow in FPA agar, since FPA cannot enter the cell, but will not grow in tryptophan agar since the *mtr* mutation will prevent the entry of tryptophan to the cell. The presence of the wild type *mtr* gene allows the growth of the *trp-2* strain in tryptophan agar and prevents the growth of the strain in FPA agar (Stadler et al. 1991; Dillon and Stadler 1994).

There are certain requirements for the correct operation of the *mtr* selection system. The host strain should carry mutations in the genes *mtr* and *trp-2* to allow the selection for loss or gain of *mtr* function. Since the host strain contains a *trp-2* mutation it is necessary to add anthranilic acid to the FPA agar to select for the loss of *mtr* function. Anthranilic acid is a precursor of tryptophan that enters the cell through an *mtr*-independent transporter and allows the growth of *trp-2* strains. There are other amino acid transporters in *Neurospora*, and tryptrophan could be transported into the mycelia of an *mtr* strain unless we add an excess of an amino acid that will compete with tryptophan for the use of these transporters. Thus, a high amount of arginine should be added to the tryptophan agar to ensure that tryptophan can only enter the cell through the MTR transporter. In addition, a high amount of arginine in the growth media seems be necessary to ensure that FPA is only transported into the cell through MTR and to allow a clean selection of the *mtr* phenotype by resistance to FPA (Linden et al. 1997, Linden and Macino 1997), but addition of arginine to the FPA agar is not strictly required (Carattoli et al. 1995; Vitalini et al. 2004).

The *mtr* selection system has been adapted for the isolation of mutants with an altered regulation of gene expression. In this case it is necessary to engineer the *mtr* gene so that the *mtr* ORF is now fused to the regulatory region of the gene under study. The resulting promoter/*mtr* fusion is now introduced into an *mtr trp-2* strain so that the *mtr* gene, and the presence of the MTR permease, is now driven by the conditions that allow the expression of the promoter/*mtr* fusion. For example, the *mtr* gene has been fused to the promoter of the light regulated *al-3* gene and introduced by transformation into an *mtr trp-2* strain. The new strain had the *mtr* gene under the regulation of a light-inducible promoter. Light exposure allowed *mtr* expression, and the entry of the toxic compound FPA, thus facilitating the isolation of blind mutants that did not express the *al-3p/mtr* fusion under light exposure, and grew on FPA agar. The blind mutants were isolated after insertional mutagenesis. The DNA inserted in

the mutant's strains allowed the cloning of the mutant gene, *wc-2* (Carattoli et al. 1995; Linden et al. 1997; Linden and Macino 1997). An alternative procedure using the *mtr* system would have been to clone the mutant genes by selecting for the gain of *mtr* function, and taking advantage of the *trp-2* mutation present in the original strain. Strains with a gain of *mtr* function could be selected by growing conidia transformed with a genomic library on tryptophan-containing agar under light exposure. Only the strains containing the complementing clone should have a light-regulated *mtr* gene that would allow the entry of tryptophan to the cell resulting in mycelial growth. This selection procedure might also allow the isolation of suppressors of the blind mutations by the use of mutagenized conidia instead of conidia transformed with a genomic library. In addition, the *mtr* system has been used to isolate mutants altered in the regulation of clock-controlled genes using strains carrying fusions of the promoters of the genes *ccg-1* and *ccg-2* to *mtr* (Vitalini et al. 2004).

Procedure

The use of the *mtr* selection system for the isolation of mutants with an altered regulation of gene expression requires the construction of a strain carrying the fusion of the promoter under study to the *mtr* gene. The materials required for these experiments are the following:

The mtr gene and strains

The sequence of the *mtr* gene (Koo and Stuart 1991, Dillon and Stadler 1994) can be found at GenBank/EMBL database (accession number L34605).

The *mtr* gene and strains carrying *mtr* and *trp-2* alleles can be obtained from the Fungal Genetics Stock Center.

The following plasmids are deposited at FGSC and contain the *mtr* gene: pCVN2.9, pN846, and pGDmtr2. A restriction map for each plasmid is available at the FGSC web site.

FGSC strains with *mtr* and *trp-2* alleles:
8288 (*mtr::hph, trp-2, A*)
8289 (*mtr::hph, trp-2, a*)
8290 (*mtr::hph, trp-2, al-2, A*)

The insertion of the *hph* gene at the *mtr* locus provides an additional marker (resistance to hygromycin) and prevents the reversion of the *mtr* phenotype.

The desired promoter fragment should be inserted upstream of the initiator ATG to drive the expression of *mtr*. A SalI site is located 40 bp upstream of the *mtr* initiator ATG, providing a useful site for promoter insertion. Other options are possible depending on the specific promoter sequence and the *mtr* plasmid being used.

Culture conditions

We use the standard Vogel's minimal agar for regular growth and sorbose agar for colony growth. The specific supplements for each type of agar are the following:

Tryptophan/Arginine (TA) agar: 0.03 mg/ml tryptophan, 0.1 mg/ml arginine

Other published compositions are:
0.01 mg/ml tryptophan, 1 mg/ml arginine (Vitalini et al. 2004)
0.01 mg/ml tryptophan, 0.1 mg/ml arginine (Carattoli et al. 1995)
0.001 mg/ml tryptophan, 1 mg/ml arginine (Linden et al. 1997)

FPA/Arthranilic acid/Arginine (FAA) agar:
0.015 mg/ml FPA, 0.05 mg/ml anthranilic acid, 0.1 mg/ml arginine

Other published compositions are:
0.06 mg/ml FPA, 0.01 mg/ml anthranilic acid (Vitalini et al. 2004)
0.015 mg/ml FPA, 0.05 mg/ml anthranilic acid (Carattoli et al. 1995)
0.05 mg/ml FPA, 0.05 mg/ml anthranilic acid, 1 mg/ml arginine (Linden et al. 1997)

These recipes are based on our laboratory experience and published work but the optimal concentration for selection may depend on each experimental set-up. We suggest that they are optimized using as starting concentrations the range described above.

Integration of the promoter/mtr fusion into an mtr trp-2 strain
The integration of the promoter/*mtr* fusion in the host strain can be obtained after selection for the active *mtr* gene if it is possible to use conditions that will activate the expression of the promoter in the promoter/*mtr* fusion. For example, the introduction of the *al-3* promoter/*mtr* fusion into an *mtr trp-2* strain was obtained by selecting for growth in TA agar under light/dark cycles that promoted a robust expression of the *al-3* promoter and the entry of tryptophan to the cell (Carattoli et al. 1995). Other alternatives are the addition of a selectable marker, like benomyl, to the *mtr* plasmid (Vitalini et al. 2004), or the co-transformation with a plasmid carrying a selectable marker. It is important to note that the strains listed here carry the *hph* gene inserted at the *mtr* gene, preventing the use of hygromycin resistance gene as a marker in co-transformation experiments.

Most of the transformants will carry ectopic integration events. It is necessary to isolate several transformants, purify them under selective conditions, and test them for growth on the appropriate selective medium (TA for expression of *mtr*, or FAA for lack of expression of *mtr*) to ensure that the promoter/*mtr* fusion behaves as expected.

Mutagenesis
Once the strain with the appropriate promoter/*mtr* fusion in an *mtr trp-2* background has been isolated and its growth phenotype tested, we need to prepare a stock of conidia for mutagenesis. There are several protocols for mutagenesis in *Neurospora*, and we prefer UV mutagenesis for the ease of handling and lack of chemical residues.

UV mutagenesis: A sample of viable conidia (5×10^6) in 0.8 ml of distilled water is placed in an empty Petri plate and kept uncovered. A sample of conidia (10 µl) should be collected before and after UV treatment to measure cell viability. The conidia are then treated under UV for the time required to obtain 50-70% survival. Lower survival rates are likely to yield a higher number of mutants but will also increase the likelihood of obtaining strains carrying multiple mutations.

After UV mutagenesis the mutagenized conidia are plated on selective agar (TA or FAA in sorbose agar) and incubated at 34ºC. Plating can be obtained by spreading 0.2 ml of conidial suspension on the surface of the selective agar or by use of spreading agar with the appropriate supplements. The handling and plating of conidia should be performed in darkness or dim light to prevent photorepair. The plates should be incubated in the dark, at least for the first 8 hour (overnight), to prevent photorepair and the subsequent reduction in UV mutagenesis. A variant of the FAA selection requires that the mutagenized conidia are incubated overnight with only anthranilic acid. FPA and arginine are added after the overnight incubation to allow the presence of the mutant proteins that prevent the expression of the promoter/*mtr* fusion (Linden et al. 1997).

Mutant colonies are picked, transferred to slants, and allowed to conidiate under selective conditions. Three cycles of vegetative growth are usually required to ensure that the resulting mycelia are homokaryotic. The mutant strains are now ready for conidial stock preparation, the assay of their phenotypes in detail, and their genetic and molecular characterization.

References

Carattoli A, Kato E, Rodriguez-Franco M, Stuart WD, Macino G (1995) A chimeric light-regulated amino acid transport system allows the isolation of blue light regulator (*blr*) mutants of *Neurospora crassa*. Proc Natl Acad Sci USA 92:6612-6616

Dillon D, Stadler D (1994) Spontaneous mutation at the *mtr* locus in *Neurospora*: the molecular spectrum in wild-type and a mutator strain. Genetics 138:61-74

Koo K, Stuart WD (1991) Sequence and structure of *mtr*, an amino acid transport gene of *Neurospora crassa*. Genome 34:644-651

Linden H, Macino G (1997) White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora* crassa. EMBO J. 16:98-109

Linden H, Rodriguez-Franco M, Macino G (1997) Mutants of *Neurospora crassa* defective in regulation of blue light perception. Mol Gen Genet 254:111-118

Stadler D, Macleod H, Dillon D (1991) Spontaneous mutation at the *mtr* locus of *Neurospora*: the spectrum of mutant types. Genetics 129:39-45

Stuart WD, Koo K, Vollmer SJ (1988) Cloning of *mtr*, an amino acid transport gene of *Neurospora crassa*. Genome 30:198-203

Vitalini MW, Morgan LW, March IJ, Bell-Pedersen D (2004) A genetic selection for circadian output pathway mutations in *Neurospora crassa*. Genetics 167:119-129