Construction of Multiple Mutant Strains by Mating Procedures for the Cloning of *pmn* and *pmb* Genes Encoding Amino Acid Permeases in *Neurospora crassa*

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The *pmb* gene encoding a basic amino acid transport protein in *Neurospora crassa* could be cloned by using a mutant strain defective in *pmb* gene as a host strain, using a negative selection on the media containing amino acid analogue canavanine. To select positive transformants of the genes for cloning, an auxotrophic marker (*his-2*) was added to a *pmb* mutant strain by mating; a triple mutant (*pmn : pmb : his-2*) was constructed by crossing a strain defective in basic amino acid transport system (*#1683-bat um 535* "A") to a double mutant strain defective in neutral amino acid transport and histidine production (*mtr6r : his-2* "a"). Crossing was performed on synthetic crossing (SC) media containing histidine. The *pmn : pmb* and *pmn : pmb : his-2* strains were selected among the progeny colonies from crosses on plates containing 50 μg/ml *para*-fluoro-phenylalanine (PFPA), 200 μg/ml canavanine, and 500 μg/ml histidine. The selected colonies were cultured on minimal media with or without histidine for discarding *pmn : pmb* strain, because the *pmn : pmb : his-2* strain grows only on histidine containing media. The *pmn : pmb : his-2* strain selected can be used as a host strain for the cloning of the *pmb* and the *pmn* genes from a *Neurospora* genomic library by means of positive selections.

Key words: mating, *pmn : pmb : his-2*, *para*-fluoro-phenylalanine, canavanine, *Neurospora crassa*, positive selection

Reproduction of *Neurospora* is either asexual or sexual. *Neurospora* has three spore forms, two asexual (micro-conidia and macroconidia) and one sexual (ascospores). Under appropriate conditions, the vegetative mycelium gives rise to a reproductive mycelium that supports the production of reproductive spores.

Similar to *Sacharomyces cerevisiae, N. crassa* has two mating types, designated A and a. A normal strain of either mating type can produce male gametes (macro- or micro-conidia) and female gametes (ascogonia, enclosed in a protective sheath, the whole structure known as a protoperithecium). Macroconidia are used to inoculate vegetative cultures, and they served as the fertilizing (male) parent in sexual crosses.

Gametic fusion occurs when conidia of one mating type are spread over the surface of a culture of the other mating type which has protoperithecia. Prior to nuclear fusion, parental nuclei divide many times as a network of ascogenous hyphae develops in the growing perithecium. At the termini of this hyphal system, nuclei of the two mating types associate in pairs in the ascus initials, where nuclear fusion occurs. The resulting diploid nucleus is immediately resolved into haploid products by two meiotic divisions and one mitotic division, which take place in the elongating ascus initial. The final product of each meiotic event is an ascus, containing eight ascospores. From each ascospore, a haploid vegetative culture is obtained (1, 2, 3).

N. crassa is known to possess at least six genetically and biochemically distinct amino acid transport systems: three constitutive amino acid permeases and three specialized amino acid permeases. The constitutive permeases include a neutral amino acid-specific system (N system), a basic amino acid-specific system (B system), and

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a general system (G system) that can transport neutral, basic, and acidic amino acids (7). The basic amino acid transport system (B system) that is involved in transport of arginine, lysine, histidine, and arginine analogue canavanine sulphate is under the genetic control of the *pmb* gene and the neutral amino acid transport system (N system) that is involved in transport of phenylalanine, histidine, and phenylalanine analogue PFPA is under the genetic control of the *mtr* gene (5, 7).

Phenylalanine passes through G system and N system. Arginine passes through G system and B system. And histidine is transported by G system, N system and B system. Therefore the excess amount of phenylalanine in media blocks N system and G system; thus, only B system is available for the transport of histidine. On the other hand the excess amount of arginine in media blocks G system and B system and only N system is available for the transport of histidine.

The cloning of *pmn* gene and *pmb* gene has been carried out on plates with PFPA and canavanine in transformation experiments by negative selections. In order to make the transformation procedures into positive selection for the cloning of *pmn* gene and *pmb* gene, we produced a new strain having *his* marker by mating procedures.

Materials and Methods

Chemicals

Para-fluoro-phenylalanine (PFPA) and canavanine were purchased from Sigma chemical company.

Strains and media

Strains used for this experiment were *#1683-bat um 535* "A" and *mtr6r : his-2* "a" which were obtained from Fungal genetic stock center and Perkins, Stanford University. Synthetic crossing (SC) medium (1 L) contains 20 ml of Vogel's 50X, 0.7 g of K_2HPO_4, 0.5 g of KH_2HPO_4, 2% of sucrose, 2% of Bacto-Difco agar, 0.1 ml of trace elements, and 2% of histidine. *Neurospora colonial* medium (25 ml) contains 0.5 ml of 50X Vogel's medium, 0.375 g of Bacto-Difco-agar, 125 μl of 100 mg/ml histidine, 1 ml of 5 mg/ml canavanine, 0.5 ml of 5 mg/ml *para*-fluoro-phenylalanine (PFPA), and 2.5 ml of 10X FIGS (fructose, inositol, glucose and sorbose media). Conidial growth medium (25 ml) contains 0.5 ml of 50X Vogel's medium, 0.5 g of sucrose, 125 μl of 100 mg/ml histidine, 0.5 ml of 5 mg/ml canavanine. Conidial growth medium (25 ml) contains 0.5 ml of 50X Vogel's medium, 0.5 g of sucrose, 125 μl of 100 mg/ml histidine.

Crossing procedures

The procedures for constructing multiple mutant strains of *Neurospora crassa,* and preparing spheroplasts, were done as described in several papers (4, 5, 6).

The genetic construction of strains was done by crossing for selection of *pmb* transport defective mutant before using this strain in transformation procedures. A strain defective in basic amino acid transport system (*#1683-bat um 535* "A"), was crossed with a double mutant strain defective in neutral amino acid transport and histidine production (*mtr6r : his-2* "a") on synthetic crossing (SC) media containing histidine. The 0.2 ml of suspension of the first parental conidia (*#1683-bat um 535* "A") was transferred onto 8 ml of SC slants containing histidine in a 18×150 mm tube as female parent, using cotton or sponge plugs. It was incubated for a week in the dark at room temperature (25℃). 0.2 ml of conidial suspension of second parent (*mtr6r : his-2* "a") was spread over the surface of the female culture. At approximately 10 days after fertilization, ascospores were collected by picking up ejected ascospores on the wall of the tube opposite the agar surface with a wet loop and suspended into a drop of water.

Isolation of the *pmn : pmb : his-2* strain

Ten days after fertilization the ascospores were picked up by a loop formed from 70% platinum and 30% iridium wire with a 0.5 mm diameter from a cross tube and suspended in 1 ml of water. The ascospore suspension was incubated at 60℃ for 30 minutes. The germinating spores were distributed evenly over a plate of 25 ml of FIGS medium containing PFPA and canavanine using a sterile glass spreader flamed in 95% ethanol. Avoid spreading the spore suspension to the extreme edge of the plate and scratching the surface. The germinating progenies were selected for both resistance to PFPA and canavanine.

A plug of cells from each colony was cored with the tip of a sterile, disposable pipette and transferred onto slants of conidial growth media with or without histidine. The selected *pmn : pmb : his-2* strain was confirmed by showing no growth in 1X Vogel's conidial growth liquid media and 100% growth in 1X Vogel's media containing histidine at 30℃ in 2~3 days.

Results and Discussion

Amino acid transport in *Neurospora crassa*

The *pmb* strains (*#1683, #2275*) are mutants defective at B system, and *pmn* strains (*mtr6r, mtr10r*) are mutants defective at N system. PFPA is phenylalanine analogue and canavanine is arginine analogue. And these chemicals are toxic to the cell due to their incorporation

in protein synthesis and thereby disrupting the structure of proteins. The experiment was carried out to test transport of amino acid analogues through the N system and the B system by means of cell growth on the plates containing analogues. The *pmb* mutants survived in the media containing canavanine and the *pmn* mutants grew in media containing PFPA.

Sensitivity of *Neurospora crassa* to amino acid analogue PFPA

Para-fluoro-phenylalanine (PFPA) is a phenylalanine analogue and toxic to the cell. PFPA is incorporated in the protein synthesis instead of phenylalanine, and thereby disrupts the protein synthesis.

A conidial suspension was incubated in a conidial growth N medium containing histidine (500 μg/ ml) and PFPA. Conidia were filtered, dried, and weighed.

The wild type strain *74a* had sensitivity to PFPA at concentration 50 μg/ml with a decrease in the growth rate abruptly. The *mtr10r* and *mtr6r* strains which are mutants defective in N system (neutral amino acid transport permease) were resistant to PFPA at concentration of up to 200 μg/ml. The strains, *#1683 bat um 535* and *#2275 bat cam-37(r)* of *pmb* mutants which have a wild type N system showed very little growth in the presence of PFPA at 50 μg/ml concentration. A 50 μg/ml concentration of PFPA was identified as suitable in experiments for selection of strain defective in N system.

Sensitivity of *Neurospora crassa* to amino acid analogue canavanine

Canavanine is arginine analogue and toxic to the cell by incorporating in the protein synthesis instead of arginine and disrupting the protein structure.

The sensitivity of a wild type strain *74a* showed high sensitivity to canavanine by a rapid decrease in the growth rate in media containing over 100 μg/ml of canavanine. The strains *#1683 (pmb)* and *mtr6r (pmn : his-2)* were used as parents in the cross for selecting *pmn : pmb : his-2* strain. The *#1683* strain was resistant to canavanine and showed over 75% growth at 200 μg/ml concentration of canavanine. The *mtr6r* strain which is pmb^+ was quite sensitive to canavanine. According to the data 200 μg/ml concentration of canavanine is suitable to use in experiments for selection of a strain which is defective in B system.

Construction of *pmn : pmb : his-2* strain

After crossing a strain defective in basic amino acid transport system (*#1683-bat um 535* "A") to a double mutant strain defective in neutral amino acid transport and histidine production (*mtr6r : his-2* "a") on synthetic crossing (SC) media containing histidine, at approximately 10 days after, ascospores were collected by picking up ejected ascospores on the wall of the tube opposite the agar surface with a wet loop and suspended into a drop of water.

Table 1. Selection of the *pmn:pmb:his-2* strain on the minimal plates containing amino acid(s).

Strains	Histidine	Histidine + Phenylalanine	Histidine + PFPA	Histidine + Canavanine
74a (wild type)	+	+	−	−
pmb (#1683)	+	+	−	+
pmn : his-2 (mtr6r)	+	+	−	−
pmn : pmb : his-2	+	−	+	+

The concentration of amino acids and analogues added in minimal media were histidine (0.5 mg/ml), phenylalanine (5 mg/ml), PFPA (0.05 mg/ml), and canavanine (0.2 mg/ml). In this condition phenylalanine blocks G system and N system, thereby only B system is available for the transport of histidine in *Neurospora crassa*. The construction of *pmn : pmb : his-2* was carried out by crossing of *pmb (#1683)* and *pmn : his-2 (mtr6r)* as described in Materials and Methods. Plus indicates cell growth, while minus indicates cell death.

N. crassa ascospores can be stored in sterile water sealed in small vials without apparent loss of viability for at least a year at room temperature and 18 months at 4℃. A convenient feature of the *Neurospora* sexual system is that dormant ascospores must be treated at 60℃ temperature for germination. The activation treatment kills parental cells and other contaminants at the same time and this allows crosses to be analyzed and selected at any time in unambiguously pure form after they are mature (1, 2, 3).

A *pmn : pmb : his-2* strain which can be used as host cells for transformation with library DNAs was produced by crossing *pmb* strain to *pmn : his-2* strain. The *pmn : pmb : his-2* strain was identified by selection for growth on histidine plates containing PFPA and canavanine in media followed by testing for growth on minimal and minimal plus histidine plates. The *pmn : pmb : his-2* strain grew on selection media and on minimal plus histidine media, but did not grow on minimal media alone.

Screening and selection of *pmn : pmb : his-2* strain

A *pmn : pmb : his-2* strain was selected among the progeny colonies from crosses between *pmb* and *pmn : his-2* parental strains on plates containing 50 μg/ml PFPA, 200 μg/ml canavanine, and 500 μg/ml histidine in media, followed by culturing on minimal colonial media with or without histidine for discarding *pmn : pmb* strain. The *pmn : pmb : his-2* strain was selected from colonies which grows only on histidine containing media.

The *pmn : pmb : his-2* strain was verified as a host strain in transformation experiments by culturing in 1X histidine plus 10X phenylalanine media. The dry weight of *pmn : pmb : his-2* strain grows fully in a medium containing histidine, but does not grow well in a medium containing histidine and 10X phenylalanine because the excess phenylalanine in the medium blocks histidine uptake via the G system. These phenotypes were resulted from the fact that histidine is transported via all three major amino acid transport systems (the B, the N, and the G system) and phenylalanine is transported by the N and the G system (7). On the other hand, wild type strain (*74a*) grew fully in a histidine plus 10X phenylalanine medium. The data of Table 1 showed very little growth of the *pmn : pmb : his-2* strain in histidine plus phenylalanine media. Thereby, this data confirm that the selected transformants were *pmn : pmb : his-2* strain which is useful as a host strain in transformation procedures for cloning the *pmn* and *pmb* strains by positive selections of transformants with pmb^+ DNA fragments.

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