Fungal Genetics Reports

Volume 39 Article 14

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Recommended Citation

Royer, J. C., and C.T. Yamashiro (1992) "Generation of transformable spheroplasts from mycelia, macroconidia, microconidia and germinating ascospores of Neurospora crassa," *Fungal Genetics Reports*: Vol. 39, Article 14. https://doi.org/10.4148/1941-4765.1440

Generation of transformable spheroplasts from mycelia, macroconidia, microconidia and germinating ascospores of Neurospora crassa

Abstract

For Neurospora to be generally useful in molecular studies it would be desirable to be able to prepare transformable spheroplasts from mycelia and any of the three types of spores produced by this organism. Transformable spheroplasts are currently prepared from germinating macroconidia by digestion with Novozym 234 in the presence of 1.0 M sorbitol (Vollmer and Yanofsky 1986. PNAS 83:4869-4873). This method is efficient, but requires a 3-5 hr germination step. Elimination of the germination step would be a technical advance. In addition, the standard method is usable only with strains that form large numbers of macroconidia. Thus, interesting mutants that are incapable of forming macro- conidia cannot be used as recipients in cloning experiments. A procedure for generating spheroplasts from mycelia of *N. crassa* has been reported (Buxton and Radford 1984. MGG 196:339-344). While large numbers of spheroplasts are released by this procedure, the frequency of transformation is low, and we have experienced difficulty obtaining repeatable results. Since we want to clone genes implicated in the macroconidiation process, we devised a procedure that improves the efficiency of transformation of mycelial spheroplasts. As an alternative approach, we developed an transformation protocol for microconidia. Since aconidial mutations can be introduced into a microcycle microconidiating background such as mcm (Maheshwari 1991. Exp. Mycol. 15:346-350), transformation of microconidia represents a viable option for the cloning of conidiation genes. A procedure for generating competent spheroplasts from germinating ascospores also was developed and provides an additional strategy for cloning conidiation genes.

Generation of transformable spheroplasts from mycelia, macroconidia, microconidia and germinating ascospores of *Neurospora crassa*

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For Neurospora to be generally useful in molecular studies it would be desirable to be able to prepare transformable spheroplasts from mycelia and any of the three types of spores produced by this organism. Transformable spheroplasts are currently prepared from germinating macroconidia by digestion with Novozym 234 in the presence of 1.0 M sorbitol (Vollmer and Yanofsky 1986. PNAS 83:4869-4873). This method is efficient, but requires a 3-5 hr germination step. Elimination of the germination step would be a technical advance. In addition, the standard method is usable only with strains that form large numbers of macroconidia. Thus, interesting mutants that are incapable of forming macro- conidia cannot be used as recipients in cloning experiments. A procedure for generating spheroplasts from mycelia of *N. crassa* has been reported (Buxton and Radford 1984. MGG 196:339-344). While large numbers of spheroplasts are released by this procedure, the frequency of transformation is low, and we have experienced difficulty obtaining repeatable results. Since we want to clone genes implicated in the macroconidiation process, we devised a procedure that improves the efficiency of transformation of mycelial spheroplasts. As an alternative approach, we developed an transformation protocol for microconidia. Since aconidial mutations can be introduced into a microcycle microconidiating background such as mcm (Maheshwari 1991. Exp. Mycol. 15:346-350), transformation of microconidia represents a viable option for the cloning of conidiation genes. A procedure for generating competent spheroplasts from germinating ascospores also was developed and provides an additional strategy for cloning conidiation genes.

In this paper, we describe protocols which generate transformable spheroplasts from mycelia, macroconidia, microconidia and germinating ascospores. The main alteration from the standard spheroplast formation protocol is the use of $MgSO_4$ (1.0 M) instead of sorbitol (1.0 M) as the osmotic stabilizer. In addition, a higher concentration of Novozym 234 (Novo Laboratories, Wilton CT, lot no. PPM 1530) and a modified culture procedure are utilized for generation of spheroplasts from mycelia. We also report on a modification of the standard spheroplast regeneration procedure which results in more rapid appearance of transformants and a 2-4 fold increase in transformation frequency of spheroplasts from germlings and mycelia.

Mycelia

1. We have used an inoculum build-up procedure to generate large amounts of actively growing mycelia. One hundred milliliters of Vogel's medium plus 1.5% sucrose (Davis and de Serres 1970. Meth. Enzymol. 17A:79-143) is inoculated with either conidia or mycelia from an agar

culture. The culture is macerated in a 360 ml Waring blender cup (two 10 s bursts with a 10 s pause), and incubated overnight on a rotary shaker (200 rpm) at 34 C. This primary culture is macerated and 30 ml are used to inoculate a secondary culture (final volume 130 ml). After overnight incubation, the secondary culture is macerated and 30 ml are used to inoculate a tertiary culture. After continued incubation (see below) mycelia from the resulting tertiary culture is centrifuged (2,000 x g, 10 min), resuspended in 30 ml of 1.0 M $MgSO_4$ and recentrifuged.

2. A crude estimate of mycelial wet weight is obtained from the difference in weights of the washed culture in the centrifuge tube and the empty tube. The culture is resuspended in approximately 2 ml of Novozym 234 (10 mg/ml in 1.0 M $MgSO_4$) per gram wet weight of mycelia. Digestion is performed at room temperature with gentle inversion on a Belly Dancer shaker (Stoval Life Science Inc., Greensboro NC) for 1 h. The resulting digest is placed on ice, and all subsequent treatments performed at 4 C.

3. The mycelial digest is filtered by gravity through 6 layers of cheesecloth placed in the bottom of a 12 ml syringe. An equal volume (approximately 15 ml) of ice-cold sorbitol (1.0 M) is passed through the syringe and cheesecloth and mixed with the spheroplast suspension by gentle inversion. Spheroplasts are sedimented at 55 x g for 10 min at 4 C. The resulting pellet is resuspended in 15 ml of ice-cold 1.0 M sorbitol and recentrifuged as above. The pellet is next resuspended in 15 ml of ice-cold STC (1.0 M sorbitol; 50 mM Tris, pH 8.0; 50 mM $CaCl_2$) and recentrifuged. The spheroplasts are finally resuspended at a concentration of 1-2 x 10(7) spheroplasts/ml in spheroplast storage buffer containing STC, PTC (40% PEG 4,000, 50 mM Tris, pH 8.0; 50 mM $CaCl_2$) and dimethylsulfoxide in a ratio of 8:2:0.1. We routinely freeze the resulting spheroplast suspension slowly by placing in a -80 C freezer.

4. Transformations are performed according to Vollmer and Yanofsky (PNAS 1986 83:4869-4873). We typically mix 10 ul of heparin (5 mg/ml in STC), 1 ug transforming DNA and 100 ul (2 x 10(6)) mycelial spheroplasts, and incubate on ice for 30 min. (Reactions containing larger amounts of mycelial spheroplasts lead to decreased transformation efficiency, even in the presence of increased heparin concentrations). One milliliter of PTC is added to the reaction mixture and incubation is continued for an additional 20 min at room temperature. Ten milliliters of regeneration agar maintained at 50 C are added to the transformation reaction, and the mixture is poured onto plating medium containing either hygromycin (250 U/ml) or benomyl (1 ug/ml). Transformants appear after 2-3 days incubation at either 30 C or 34 C.

The formula for regeneration (top) agar is:

```
      per liter

Vogel's 50x   20 ml
water        825 ml           After autoclaving, add
sorbitol     182 g (1 M final)
agar          15 g            1FGS additive (10x) - 100 ml

The formula for plating medium (bottom agar) is:
      per liter
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Vogel's 50x  20 ml            Autoclave and add, before pouring
water       880 ml
agar         15 g             FGS additive (10x) - 100 ml
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1FGS contains, per liter: 5 g fructose, 5 g glucose, 200 g sorbose

Using the above procedure, we have generated approximately 5 x 10(7) spheroplasts from either 3, 4 or 6 h tertiary cultures of the wild-type strain (74-OR23-1A), and 5 x 10(8) spheroplasts from a 9 h culture. Osmotically sensitive spheroplasts have been obtained from overnight cultures of wild-type, but these proved to be less competent. With mycelial spheroplasts we typically observe regeneration frequencies of about 10%. Approximately 50 stable, benomyl resistant transformants have been obtained from 10(6) spheroplasts using 0.5 ug of either pBT3 or pMO63 (Selitrennikoff and Sachs 1991, FGN 38:90-91). Efficiency of transformation with the hygromycin resistance plasmid pMP6, which contains the hygromycin phosphotransferase gene driven by a modified *cpc-1* regulatory region (M. Plamann, pers. comm.) was approximately 10-fold lower. We have obtained similar transformation frequencies using mycelial spheroplasts generated from the aconidial mutant *acon-3* (Matsuyama et al. 1974 Dev. Biol. 41:278-287).

Macroconidia

1. Macroconidia from a 2-3 week agar culture grown at 22 C are suspended in sterile water, filtered through cheesecloth, and centrifuged for 10 min at 800 x g. The conidia are then resuspended in 30 ml of 1.0 M $MgSO_4$, sedimented as above, and resuspended in 10 ml of $MgSO_4$ per 1 x 10(9) conidia.

2. Two milliliters of Novozym 234 (5 mg/ml in 1.0 M $MgSO_4$) are added per 1 x 10(9) conidia, and the conidial suspension is incubated on a rotary shaker (100 rpm) at 30 C until osmotically sensitive cells are generated (typically 30-60 min). The reaction is placed on ice, and all subsequent procedures are performed at 4 C.

3. An equal volume of ice-cold 1.0 M sorbitol is added to the spheroplast suspension, and the mixture is centrifuged at 500 x g for 10 min at 4 C. The resulting pellet is resuspended in 15 ml of 1.0 M sorbitol and recentrifuged as above. The pellet is next resuspended in 15 ml of STC and recentrifuged. Resulting spheroplasts are finally resuspended at a concentration of 1 x 10(8)/ml in spheroplast storage buffer. (Some clumping of spheroplasts is typically observed at this step). Aliquots of the competent spheroplasts are dispensed into microcentrifuge tubes and slowly frozen by placing in a -80 C freezer.

4. Transformation reactions are performed as described for mycelial spheroplasts, except that 1 x 10(7) conidial spheroplasts are used per transformation reaction.

Using the procedure described, we typically obtain between 100 and 1,000 hygromycin-resistant transformants using 1 x 10(7) spheroplasts from a variety of strains and 1 ug of pMP6. Approximately 25% of the transformants are stable when transferred to fresh hygromycin medium. Transformation efficiencies using pDH25 and pCSN43 (Staben et al. 1989 FGN 36:79-81), which contain the hygromycin phosphotransferase gene driven by the *trpC* promoter of *Aspergillus nidulans*, were dramatically reduced compared to pMP6. Transformation to benomyl

resistance with pBT3 (Orbach et al. 1986 Mol. Cell Biol. 6:2452-2461) was unsuccessful. We have generated transformable spheroplasts from either fresh conidia as described above, or conidia stored in water at 4 C for 1 or 2 days.

Microconidia

Microconidia from *mcm* are generated as described by Maheshwari (1991) Exp. Mycol. 15:346-350) by inoculating 100 ml Vogel's medium + 1.5% glucose in a 500 ml Erlenmeyer flask with 5 x 10(8) macroconidia and shaking at 240 rpm, 22 C for 16-24 hr. After filtration through cheesecloth (which generates a filtrate consisting of >99% microconidia) the microconidia are pelleted by centrifugation (800 x g, 10 min) and resuspended in 50 ml 1 mM EDTA pH 8.0, 25 mM 2-mercaptoethanol. The microconidia are shaken (100 rpm) for 20 min at 30 C and then pelleted by centrifugation (800 x g, 10 min). (It is uncertain whether this pretreatment is necessary for spheroplast formation). From this point on, the procedure described above for spheroplasting and transforming macroconidia is followed (steps 2-4). Spheroplast viability was ~12% and 300-500 transformants were obtained when 1 ug of pMP6 and 10 ul of 5 mg/ml heparin were mixed with 1 x 10(7) spheroplasts.

Germinating ascospores

1. Ascospore production is initiated by inoculating the female parent onto the center of 150 mm petri dishes containing synthetic crossing agar (Davis and de Serres 1970 Meth. Enzymol. 17A:79-143) and incubating at 25 C for 7-10 days with a 12 hr light/dark cycle. Conidia (1 x 10(6)) are added to each plate for fertilization. The plates are then incubated at 25 C for an additional 3 weeks. (Plates are inverted in the dark for the final two weeks). Ascospores are collected off the lids of the petri dishes by scraping in sterile H_2O with a rubber policeman and pelleted by centrifugation (800 x g, 5 min). A typical yield is 1 x 10(8) ascospores per plate.

2. Ascospores are resuspended in 10 ml Vogel's medium, transferred to a 250 ml Erlenmeyer flask and activated by heat shocking at 60 C for 45 min. Following this treatment, ascospores are added to 140 ml of Vogel's medium and germinated by shaking at 200 rpm, 30 C for 4-7 h. (We have observed that germination, which occurs from both ends of the ascospore, is not as synchronous as conidial germination). Ascospore germlings are collected by centrifugation (800 x g, 5 min) when 30-75% of the spores have germinated, and germ tubes are 1-5 ascospore lengths.

3. Germlings are washed once in 20 ml ice-cold 1.0 M sorbitol and then resuspended in 10 ml ice-cold 1.0 M sorbitol. Spheroplasts are prepared by adding 2 ml 5 mg/ml Novozym 234 (in 1.0 M sorbitol) and then shaking gently (100 rpm) for 30-60 min at 30 C. (The dewalled germ tubes often detach from the ascospore shell during this step). The spheroplasts are then washed and prepared for transformation as described above for macroconidia starting at step 3.

Using pBT3, 10-900 transformants per microgram DNA per 5 x 10(6) spheroplasts have been obtained. The transformation efficiency appears to vary widely with different parental genotypes. Transformations with plasmids containing hygromycin-resistance genes resulted in very few, if any, transformants.

Alternate spheroplast regeneration protocol

We have developed a modified regeneration procedure which uses sucrose as the osmotic stabilizer and tergitol (Springer 1991 FGN 38:92) to promote restricted, colonial morphology. Bottom agar contains Vogel's salts (1x), sucrose (15 g/L), tergitol (0.005%) and the selection drug. Regeneration top agar (used at 10 ml/plate) contains Vogel's salts (1x) and sucrose at 0.6 M. We have observed a 2-4 fold enhancement in spheroplast regeneration and transformation efficiency using spheroplasts from germlings and mycelia. When identical aliquots from a single transformation reaction containing wild-type germling spheroplasts and pBT3 were plated on the two different regeneration media, 253 +/- 7 (S.E.) benomyl resistant transformants were obtained on the sucrose/tergitol medium, while 61 +/- 10 transformants were obtained using the standard regeneration medium. Dilutions of the same experiment plated on non-selective media resulted in 19.0 +/- 3.2 regenerates on sucrose/tergitol medium and 9.7 +/- 1.7 regenerates on the standard regeneration medium. In addition, transformants were evident after 24 hr at 30 C using the sucrose/tergitol medium, but did not appear until 48 h incubation using the standard regeneration medium. A potential problem with the new method is that the colonial growth morphology is lost when the colonies emerge from the growth medium. As a result, the number of transformants per plate must be reduced, and transformants must be transferred after 2 days at 30 C. The benefit of increased efficiency must be balanced with the less restricted growth morphology. We have not determined the efficiency of the modified regeneration procedure using spheroplasts from conidia or ascospores.

We wish to acknowledge the support of Charles Yanofsky and David Perkins. CTY was supported by a fellowship from the American Cancer Society (PF-3509). These studies were supported by a grant from the U.S. Public Health Service (GM41296).