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Simple and Reliable Method for Replica Plating *Neurospora crassa*[1]

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The details of a simple method for replica plating *Neurospora crassa* are described. The procedure has proved to be highly reliable for replica plating of colonies arising from either ascospores or conidia.

Replica plating, as first described by Lederberg and Lederberg (11) for use with bacteria, has proved to be a valuable technique in studying the genetics of a number of microorganisms. The ability to screen rapidly large numbers of colonies is vital in many mutational and recombinational studies. The simplicity of the Lederbergs' procedure has made such screening feasible for most bacterial species and for some eukaryotic species as well (6, 13).

Generally, however, the bacterial replica plating procedure has not worked well with filamentous fungi, such as *Neurospora crassa*. Except in special cases (14, 20), it has failed to meet one or both of the basic requirements for a successful replica plating procedure. First, the procedure must reliably pick up a portion of each colony from the master plate and transfer it to a fresh agar surface. Second, the procedure must not result in the establishment of any significant number of spurious colonies on the replicate plate(s). We have developed a simple replica plating technique for use with *Neurospora crassa* which meets these requirements and which we believe will be of general usefulness.

MATERIALS AND METHODS

Strains. The *N. crassa* strains were all constructed in this laboratory from strains originally received from either Adrian M. Srb (Cornell University, Ithaca, N.Y.) or the Fungal Genetics Stock Center (Humbolt State College, Arcata, Calif.). The following mutations were utilized (the designations in parentheses indicate allele number, if known, and linkage group): *cot-1*, colonial temperature-sensitive (C102t, IVR); *gul-1*, gulliver (RL2068, VR); *ad-3B*, adenine (C-18-972, IR); *arg-5*, arginine (27947, IIR); *leu-3*, leucine (R156, IL); *lys-1*, lysine (33933, VC); *inos*, inositol (37401, VR); *me-3*, methionine (VR); *pab-2*, *p*-amino-benzoic acid (VR); and *am*, α-amino nitrogen (47305, VR).

Media. The minimal medium in these experiments is essentially that of Beadle and Tatum (1), commonly known as Fries' medium. The carbon source in glucose minimal is 2% D-glucose. The carbon source in sorbose minimal is 1% L-sorbose plus 0.025% D-glucose plus 0.025% D-fructose, which is autoclaved separately from the salts solution. Prior to each plating experiment, cultures were grown on YEGCE medium (9), a rich medium containing 1% D-glucose as a carbon source. Conidia from these cultures were used for the platings. The crossing medium used was 3X corn meal agar (8). All media were solidified with 1.5% agar (Difco). When appropriate, the minimal media were supplemented with the indicated amounts of biochemicals, all of which were obtained from Sigma Chemical Co.

Crossing and plating methods. The procedures used to make crosses and to plate either conidia or ascospores were essentially those described by Davis and deSerres (5).

Replication. The general procedure for replica plating is described below. Some possible variations will be referred to in the Discussion section.

Either conidia or heat-shocked ascospores were spread over the master plates (plastic petri dishes; 100 by 15 mm; internal diameter, 87 mm) containing appropriately supplemented sorbose minimal medium. Unless otherwise specified, the conidia were from strains carrying the *cot-1* mutation (15, 16), and the ascospores were from crosses that were homozygous for this mutation. A mark was placed on the side of each petri dish bottom with a felt-tip pen for orientation. After these plates were incubated overnight at 30 C, each was covered with one or two sterile discs, 79 mm in diameter, of Whatman no. 1 filter paper (W. & R. Balston, Ltd., Maidstone, England). The use of two filter-paper discs has proved to be feasible (i.e., the top disc is as good as the bottom disc with respect to reliability of replica transfer) and reduces the total time required to carry out the entire replication procedure. When two discs were used, the master plates were inspected about 10 min after the filter paper was placed over the agar sur-

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face. Any air bubbles that remained under the top disc were teased out with a small, sterile glass spreader (ca. 25 mm across). The filter-paper discs were left on the master plates for 24 to 30 hr at 30 C to allow the colonies to grow into the paper. Just before the discs were transferred for the first time, orientation marks were placed on each disc by tearing out a very small piece of the disc with a pair of sterile sharp-pointed forceps (e.g., Fisher catalog no. 8-880), beside the mark on the petri-dish bottom. Then each disc was picked up with the forceps and transferred to fresh plates of appropriately supplemented glucose minimal. This transfer was performed by picking up each disc on one side and slowly peeling it either from the underlying disc or from the agar surface and laying it on the surface of a fresh plate. The positioning of the disc may be accomplished more easily if the plate is held about 60° from horizontal. After the disc was transferred to each replicate plate, an orientation mark was placed on the side of the plate with a felt-tip pen, beside the tear in the disc.

After the discs were transferred, the replicate plates were incubated at 38 C for 1 day. The master plates were incubated at 30 C one more day and stored at 4 C until all plates were ready to be examined. The transfer may be repeated several times by moving the same filter paper from one replicate plate to another if sufficient time is allowed for the hyphae to grow from the paper into the agar. We allowed 24 hr for each plate; 12 hr may be sufficient for most strains. The replicate plates were incubated at 38 C for a total of about 3 days, at which time they could be easily scored. They may then be stored at 4 C if necessary. Very little growth occurs on the glucose minimal plates at 4 C; we have stored plates at this temperature for several weeks and still had distinct colonies. The sorbose minimal master plates may be stored several months at 4 C.

If possible, all phases of the replication procedure should be carried out in a sterile transfer chamber, free from air currents.

Scoring. The pattern of colonies on each master plate was compared with the pattern on its replicates. In some instances, a negative result consisted of a faint outline of the colony; in others, no growth was observed. A set of control plates was always included in each experiment in which the last replication was that to glucose minimal medium plus all appropriate supplements. These controls allow one to distinguish a real negative result from a failure of the replica plating procedure. A Quebec colony counter equipped with a 1.5× magnifying lens was used to aid in the scoring.

RESULTS

Application of method. Filter-paper replication has been used successfully in our laboratory for colonies arising from either *N. crassa* ascospores or conidia. The application of this technique to the scoring of ascospore colony phenotypes is demonstrated by a cross between *A; cot-1*$^-$*; lys-1*$^+$, *inos*$^-$ and *a; cot-1*$^-$*; lys-1*$^-$, *inos*$^+$ strains. Thirty-seven days after the cross was made, ascospores were collected, heat-shocked, and plated on sorbose minimal medium supplemented with low concentrations of lysine and *myo*-inositol. Colonies on these plates were replicated as described above. The four possible phenotypes (excluding mating type differences) were observed in the following numbers among 468 colonies: 156 lysine$^+$ inositol$^-$, 126 lysine$^-$ inositol$^+$, 95 lysine$^-$ inositol$^-$, and 91 lysine$^+$ inositol$^+$. The recombination observed between *lys-1* and *inos* was 39.7%. These loci were previously reported to be located on linkage group V, approximately 39 map units apart (5).

Figure 1 shows one set of master and replicate plates from this analysis.

Reliability of method. We have found this method to be highly reliable for use with *N. crassa*. In the analysis of the above cross, we observed transfer to the final replicate plate (glucose minimal plus lysine plus *myo*-inositol) for 97% of all colonies which were covered by the filter-paper discs on the master plates. All 15 failures were apparently due to extraordinarily slow germination. We found no spurious colonies on any of the replicate plates.

Appropriate levels of supplementation in master plates. Since filter paper is absorbent and contacts the surface of the master plate for some time, it may transfer nutrients from the master to the replicate plates. The supplemental concentration of the master plates must be sufficient to support the growth of auxotrophic strains but not so great that enough supplement is transferred to the replicate plates to blur distinctions between auxotrophic and prototrophic colonies. An empirical rule is to use the lowest concentration of supplement that will produce the largest possible colony diameter in an auxotrophic *cot-1*$^-$ strain after 5 days of incubation at 38 C. We have determined this concentration for a small number of auxotrophic mutants (Table 1).

DISCUSSION

Principles of the technique. In the method of replication described here, replica transfer is achieved by hyphal growth. Growth first proceeds from the master plate to the filter paper(s), then from the filter paper(s) to each replicate plate. Our development of this procedure was based upon the brief description by Reissig (18) of a replica plating method that used pipe-paper as the transfer agent.

The success of filter-paper replication in *N. crassa* depends upon the maintenance of small distinct colonies, whereas the normal growth habit is that of unrestricted spreading growth.

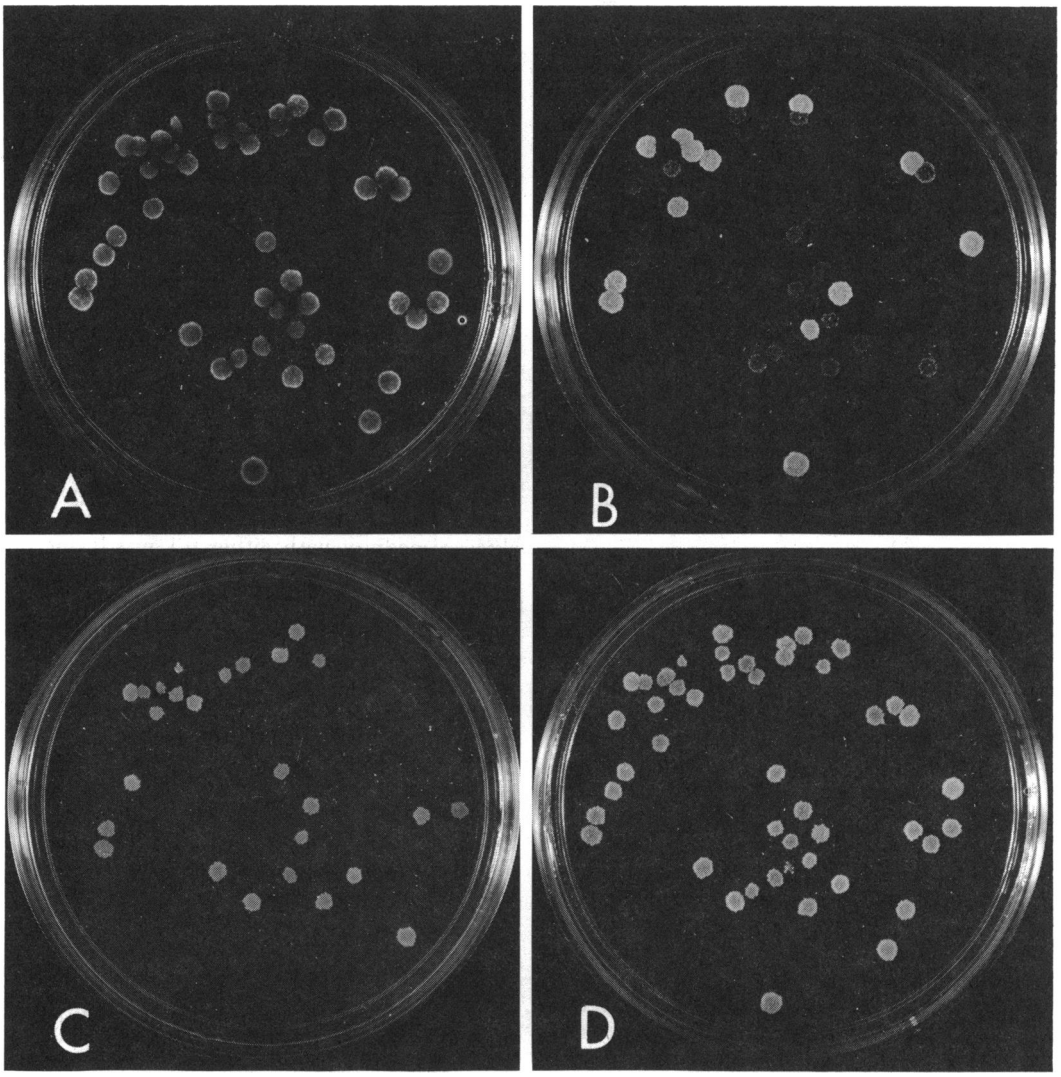

FIG. 1. *Typical N. crassa master plate with three replicates. Ascospores from cross RL3591 (A; cot-1⁻; lys-1⁺, inos⁻ × a; cot-1⁻; lys-1⁻, inos⁺) were plated on sorbose minimal containing 0.028 mM myo-inositol and 0.34 mM lysine (A) and incubated at 30 C. Replicas were transferred by the procedure described in the text to glucose minimal containing 3.4 mM lysine (B), to glucose minimal containing 0.28 mM myo-inositol (C), and to glucose minimal containing 0.28 mM myo-inositol and 3.4 mM lysine (D). The replicate plates were incubated at 38 C. Forty-seven colonies were scored on this set of plates.*

We have used two different means to enforce colonial growth. On the master plates, growth is restricted by the inclusion of L-sorbose and an accompanying decrease in the concentration of the normal carbon source in the medium (3, 21). On the replicate plates, growth is restricted by the action of *cot-1⁻* (15, 16) at high incubation temperatures (34–40 C). The correct classification of the nutritional requirements of auxotrophic colonies also depends upon the correct choice of supplement concentration for the master plates.

The use of L-sorbose for colonial restriction on the master plates and of *cot-1⁻* for colonial restriction on the replicate plates has proved to be a particularly good combination. By plating either ascospores or conidia on sorbose minimal at 30 C, one obtains visible colonies much sooner than if the plating were done on glucose minimal medium at 38 C. A second advantage is that the spread of *cot-1⁻* colonies ceases after the shift from 30 to 38 C incubation, whereas the colonies continue to enlarge on sorbose media at 30 C. A third advantage is

TABLE 1. *Appropriate supplementation[a] concentrations for master plates*

Genotype of strain tested	Normal supplementation concn (mM)[b]	Suggested supplementation concn (mM)[c]
cot-1⁻; ad-3B⁻	1.5	0.015
cot-1⁻; am⁻	1.4[d]	1.4[d]
cot-1⁻; arg-5⁻	2.9	0.090
cot-1⁻; inos⁻	0.28	0.028
cot-1⁻; leu-3⁻	1.5	0.15
cot-1⁻; lys-1⁻	1.7	0.17
cot-1⁻; me-3⁻	3.4	0.06
cot-1⁻; pab-2⁻	0.015	0.0005

[a] Adenine is supplied as adenine sulfate; amino acids are supplied in the L-form.

[b] Concentration ordinarily used in our laboratory to support the growth of auxotrophs.

[c] Lowest concentration which resulted in the maximum possible colony diameter for this strain after 5 days of incubation at 38 C.

[d] Glutamic acid.

that *cot-1⁻* colonies do not conidiate when incubated for up to 3 weeks at 38 C, whereas sorbose-restricted colonies (either *cot-1⁻* or *cot-1⁺*) conidiate within approximately 5 days at 30 C. This fact virtually eliminates the occurrence of spurious colonies on the replicate plates and allows one to make a larger number of replicate transfers than would be possible if only 30 C sorbose minimal were used.

We believe that the method of replication described here is of more general usefulness than those described previously for *Neurospora* (2, 4, 14, 17, 20) and for other filamentous fungi (10, 12, 19). The attractiveness of filter-paper replication lies in its reliability and simplicity. The necessary supplies are inexpensive and normally available in laboratories which use *Neurospora*, and all steps in the procedure are very easy to perform.

Experience with various cot mutations. When grown on solid medium at 20 to 28 C, strains carrying the C102*t* mutant allele at the *cot-1* locus are indistinguishable from wild-type strains with respect to gross morphological features and conidial production. They are quite satisfactory for general laboratory use.

There are four other colonial temperature-sensitive mutations currently available in *Neurospora* (7). Our strains carrying *cot-3⁻* do not grow well enough for replica plating usage, whereas the growth of *cot-4⁻* strains is not sufficiently restricted at high temperatures to make their use practical for this procedure. We have not extensively tested strains carrying either *cot-2⁻* or *cot-5⁻*.

Terenzi and Reissig (22) described a number of "gulliver" mutations which modify the colony size of *cot-1⁻* strains incubated at high temperatures. We would recommend that one normally avoid such mutations in strains being used for replicate plating. Colonies of *cot-1⁻; gul-1⁻* strains grow to a diameter of 10 to 15 mm on glucose minimal at 38 C and begin to conidiate within 4 days after inoculation.

Modifications of method. In certain situations, it may be necessary to modify the procedure so that only one or the other means of colonial restriction is used. A case in point is the *leu-3⁻* mutation. Strains carrying this mutation grow well on glucose minimal plus leucine but grow quite poorly on sorbose minimal plus leucine, regardless of the concentration of leucine (R. K. Littlewood, *unpublished data*). In such cases, where one must rely solely on *cot-1⁻*-induced growth restriction, it would be advantageous to supplement the glucose medium with either 0.1 mM lysine or arginine. These two amino acids stimulate the growth of *cot-1⁻* strains on glucose media at high temperatures (R. K. Littlewood, *unpublished data*).

In cases where one must rely solely on sorbose-induced colony restriction, changes in the replicate plate media may be helpful in enhancing growth restriction (3). It may be advantageous either to substitute the salt solution of Westergaard and Mitchell's synthetic crossing medium (5) for that of Fries' medium in the replicate plates or to increase the L-sorbose concentration.

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