Putative transformation of Neurospora os-1 protoplasts by a plasmid containing a G-481 phosphotransferase gene

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Putative transformation of Neurospora os-1 protoplasts by a plasmid containing a G-481 phosphotransferase gene

Abstract
Putative transformation of Neurospora os-1 protoplasts by a plasmid containing a G-481 phosphotransferase gene
Selitrennikoff, C. P.  
The formation of stable protoplasts from os-1 (NM33t) (FGSC #4493 or 4494) mycelial cultures involves the germination of macroconidia in Vogel's medium N containing 10% sorbose and 200-400 µg/ml purified Polyoxin B at 37° C (Selitrennikoff et al., 1981 Exper. Mycol. 5: 155-161). Polyoxin B is purified from Polyoxin AL Wettable Powder (Kaken Chemical Co.) by Norite and Dowex chromatography. Although not overly difficult, complete purification is time consuming. As an alternative, partially purified (40%) polyoxin was used and found to be as effective in promoting protoplasts as was purified (b 70%) polyoxin. Simply, AL Wettable Powder is made to 10% in water (100 mls), the insoluble material filtered using Whatman #1 paper and the dark brown filtrate adsorbed to a granular Norite column, washed with H2O, filtered, sterilized, and stored frozen at -20° C until used (up to six months without detectable loss of activity). A granular Norite column is extremely fast flowing so that the entire partial purification procedure requires less than a day. If the Norite column step is omitted, i.e., if crude polyoxin is used, then protoplast formation is inhibited. (Supported by National Science Foundation award PCM 8112212.)

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Transformation of filamentous fungi (including Neurospora) using a number of selectable markers has been reported (Stohl and Lambowitz, 1983 Proc. Natl. Acad. Sci. USA 80: 1058-1062; Case et al., 1979 Proc. Natl. Acad. Sci. USA 76: 5259-5263). This includes the use of plasmids carrying an appropriate gene into an auxotrophic host, as well as a variety of other selectable markers. Hosts typically are cells whose cell walls have been removed by treatment with wall-degrading enzymes and, after treatment, are allowed to regenerate cell walls (e.g. Case et al., ibid).

In this communication we report the apparent transformation of a temperature-sensitive protoplast-forming os-1 strain of Neurospora crassa by plasmid pSV-3 neo. This plasmid contains a gene that codes for a phosphotransferase (Southern and Berg, 1982 J. Mol. Applied Genetics 1: 327-341) thus conferring resistance to Kanamycin or Antibiotic G-418. os-1 cells are sensitive to G-418 (see below), yet after treatment with purified pSV-3 neo plasmid DNA, G-418-resistant clones are recovered. The precise fate of the pSV-3 neo DNA is not known with certainty, yet available data are consistent with the idea that the plasmid (or at least the gene coding for the phosphotransferase) remains autonomous.

Protoplasts of os-1, nic-1 were formed and grown as described (Selitrennikoff et al., 1981 Exper. Mycol. 5: 155-161). Cells (protoplasts) were harvested from log phase cultures by centrifugation (500 x g; 5 min) and washed twice with 50 mls ice-cold medium (SS: 7.5% (w/v) sorbitol, 1.5% (w/v) sucrose, 1X Vogel’s Salts, 10 µg/ml nicotinamide). Plasmid DNA was purified from an E. coli strain containing pSV3 neo by ethidium bromide - CsCl density centrifugation.

Purified supercoiled DNA (~ 4 µg) was mixed with ~ 15 µg high molecular weight salmon sperm DNA (carrier) in 250 µl buffer containing 145 mM NaCl, 21 mM HEPES, 0.7 mM NaH2PO4, pH 7.1. To this mixture 12.5 µl 2.5 M CaCl2 was added and a precipitate allowed to form for 30 min at ambient temperature. This mixture was added to an os-1 cell-pellet containing ~ 5 x 107 cells, and incubated for 15 min at ambient temperature. Fresh medium (10 ml) was added and the culture shaken for 2 h at 25° C. Cells were shocked with glycerol (3 ml for 60 sec), 20 mls fresh medium added and cells harvested by centrifugation. Cell pellets were resuspended in warm (45° C) SS medium containing 1.0% agar and poured into petri dishes (final cell concentration 1 x 106 cells/ml). After 24 h incubation at 25° C, cells were overlaid with SS + 1.0% agar containing antibiotic G-418 (final concentration, 400 µg/ml). After seven days of incubation at 25° C, colonies were transferred to individual plates containing 200 µg/ml antibiotic G-418. No G-418 resistant colonies were recovered in control experiments (< 1 per 106 cells). About 40 colonies were recovered using 4 µg plasmid DNA. In each case resulting cultures had formed hyphae and macroconidia. To convert resultant conidia to protoplasts, conidia were harvested and germinated in Nelson's liquid medium A (10% (w/v) sorbose, 2% (w/v) sucrose) containing 200 µg/ml Polyoxin B and 200 µg/ml G-418 at 37° C. After 2 days of incubation with shaking, resulting protoplasts were transferred and maintained in SS + 200 µg/ml G-418 at 37° C.
TABLE I

Effect of G-418 on growth of os-1 protoplasts

| STRAIN       | G-418 Concentration µg/ml | Time of incubation (Hours) |
|--------------|---------------------------|----------------------------|
|              |                           | 2  | 6  | 18 | 22 |
| Control      |                           | 0  | 6b | 11 | 103| 123 |
| 50           |                           | 5  | 10 | 45 | 63 |
| 100          |                           | 6  | 9  | 21 | 28 |
| 200          |                           | 4  | 7  | 18 | 23 |
| Transformed  |                           | 0  | 5  | 15 | 113| 123 |
| 200          |                           | 4  | 10 | 84 | 96 |
| 400          |                           | 3  | 9  | 58 | 72 |
| Transformed  |                           | 0  | 3  | 16 | 114| 130|
| -G418        |                           | 200| 2  | 6  | 28 | 37 |

aProtoplasts of os-1, nic-1 were inoculated at about x 10^5 cells/ml and their growth (Klett units) recorded at the indicated times of incubation (SS medium containing the indicated final concentration of filter-sterilized G-418) at 37°C with shaking (140 rpm).

Putative transformed cultures were tested for their sensitivity to G-418. The results presented in Table I show that control cultures were inhibited by as little as 50 µg/ml (50% inhibition) while 100 and 200 µg/ml inhibited growth by ~80%. The results using a putatively transformed strain are shown in Table I (Transformed). In contrast to the untreated controls, these cells were about 4-fold more resistant to G-418. When resistant cells were cultured for 7 days in the absence of G-418 and then retested for sensitivity, they were found to have lost their resistance (Table I, transformed-G-418). Further, the G-418-resistant phenotype was not recovered in the progeny of backcrosses to 74-OR23-1A wild-type (five putative transformed strains tested).

Overall, our data suggest successful transformation of os-1 protoplasts by pSV-3 neo DNA. The definitive proof will, of course, be the demonstration of plasmid sequences in Neurospora. We hope that our preliminary work will encourage others to examine this system.  

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