Heat Shock at an Elevated Temperature Improves Transformation Efficiency of Protoplasts from *Podospora anserina*

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We have developed an improved transformation procedure for the filamentous fungus *Podospora anserina*. This procedure is based on the observation that a heat shock at an elevated temperature (48 °C) improves the competence of *P. anserina* protoplasts for transformation 5- to 10-fold. This is observable only if the heat shock is applied before the addition of transforming DNA. An increase in competence is observed immediately after the heat shock, and heat-shocked cells are still competent after 20–30 min. The mechanism by which heat shock improves competence remains unclear. The modified transformation procedure gives as many as 200–500 stable transformants per pg of plasmid DNA containing the *P. anserina ura5* gene. This should allow direct cloning of *P. anserina* genes from a cosmid library.

**INTRODUCTION**

Fungi are widely used in biotechnology and represent a potent genetic tool for studying multicellular eukaryotes. The lack of efficient transformation systems has hampered progress in the study of fungi. Recently, efficient transformation procedures have been developed in *Neurospora crassa* using spheroplasts from germinated conidiospores (Case et al., 1979; Akins & Lambowitz, 1985) and in *Aspergillus nidulans* using a vector containing the particular *ansl* sequence whose function is still unknown (Ballance & Turner, 1985). An autonomous replicating vector has also been reported to give a high number of transformants in *Mucor circinelloides* (Van Heeswijk, 1986). Successful transformation has been reported in many other fungi (Ballance, 1986; Munoz-Rivas et al., 1986; Sanchez et al., 1987; Rodriguez & Yoder, 1987) but the number of transformants generally obtained is very low (about 10–50 per μg of plasmid DNA). Protoplasts from mycelium are easily obtained in large amounts using cell wall cellulases but they are generally poorly competent. Competence has been reported to vary greatly with the source of cellulases and with the batch of enzyme used (Akins & Lambowitz, 1985). The physiological state of the mycelium used to produce the protoplasts is also important. Other factors that influence transformation have been studied in *N. crassa* (Buxton & Radford, 1984) and in *A. nidulans* (Turner & Ballance, 1985). They include the purity of the plasmid preparation, and the quality of buffers, CaCl₂ and polyethylene glycol (PEG). Moreover the best results are obtained when transformed cells are spread embedded in an overlay of the selective medium.

Successful transformation of a *ura5* mutant has been previously described in the filamentous fungus *Podospora anserina* (Begueret et al., 1984). Efficiency was about 5–20 transformants per μg of plasmid DNA, which did not permit cloning genes of *P. anserina* directly by complementation. Here we report the effect of a heat shock treatment at an elevated temperature on the competence of *P. anserina* protoplasts.

**METHODS**

*Podospora anserina* and *Escherichia coli* strains. Wild-type *P. anserina* was strain s isolated by Bernet (1965). The isolation and characterization of a *ura5* mutant deficient in orotidylate acid pyrophosphorylase (OMPppase) have
been described previously (Razanamparany & Begueret, 1986). Transformation of a Leu- strain with the su8 suppressor gene has been reported (Brygoo & Debuchy, 1985).

Plasmids were propagated in E. coli strain B15183 (endA sbcB recBC galK met Ste8 thi-1 bioT hsdR).

Plasmids. The plasmid pPAura5-6 used in the transformation of the auxotrophic ura5 mutant is derived from a tandem insertion into pBR322 of the 1.55 kb EcoRI-EcoRI fragment carrying the ura5 gene of P. anserina (Begueret et al., 1984). The plasmid pSu8, carrying the su8 tRNA gene that suppresses a Leu- opal mutation of P. anserina, was kindly provided by Y. Brygoo. The plasmid pBT3, conferring resistance to benomyl upon transformed protoplasts (Orbach et al., 1986), was generously given by Dr C. Yanofsky. Plasmids were isolated by two CsCl/ethidium bromide density-gradient centrifugations of cleared lysates (Clewel & Helinski, 1972).

Standard transformation of protoplasts. Protoplasts were prepared from the mycelium of 36-h-old cultures grown in liquid medium supplemented with uridine. The mycelium was washed in 25 mM-potassium phosphate pH 5.8/0.8 M-sorbitol buffer and cell walls were digested in the same buffer containing 80 mg β-D-glucanase (‘Glucanex’; Novo Ferments, Basel, Switzerland) per g of mycelium. Protoplasts were separated from mycelial debris by filtration, collected by centrifugation and suspended in ST washing buffer (0.1 M-Tris/HCl pH 7.5, 0.8 M-sorbitol). The protoplasts were pelleted again and resuspended in ST buffer containing 50 mM-CaCl2 at a concentration of 106 cells ml-1. DNA (1–10 μg in 5 μl H2O) was mixed with 50 μl of protoplasts (5 × 107 cells) for 10 min at room temperature, and then 500 μl of a solution containing 60% (w/v) PEG 4000, 0.01 M-Tris/HCl pH 7.5 and 50 mM-CaCl2 was added. After 10 min at room temperature, 450 μl ST buffer containing 0.01 M-CaCl2 was added. Transformed protoplasts were separated at 45 °C in melted selective medium containing 0.8 M-sucrose and 2% (w/v) agar and overlaid onto the same medium.

In the experiments including a heat shock, protoplasts were treated at the desired temperature for 5 min, cooled on ice for 30 s and incubated at room temperature for 5 min before the addition of DNA.

RESULTS AND DISCUSSION

Protoplasts from mycelium of P. anserina can be obtained repeatedly in large amounts using Glucanex, which is a fungal cellulase preparation used to eliminate glucans in some wine preparations. Our standard transformation protocol includes incubation of protoplasts with DNA in the presence of CaCl2, treatment of the cell suspension with PEG 4000 and spreading of the transformed cells on a selective medium. We have found that optimal concentrations are 50 mM for CaCl2 and 60% (w/v) for PEG. Spreading of the transformed cells embedded in an overlay of selective medium containing 2% (w/v) agar maintained melted at 45 °C significantly improves the transformation yield. The quality of the plasmid DNA preparation is crucial: plasmids prepared by two CsCl centrifugation steps were 5–10 times more efficient in transformation than those obtained after one such step.

A new protocol including a heat shock treatment was tried to improve the transformation efficiency, as is done with some bacteria and plants. Protoplasts were heat shocked for 5 min at the temperatures indicated in Fig. 1. Plasmid DNA was added 10 min later and the cells were treated with 60% PEG 4000 as described in Methods. Survival of the cells was checked by plating appropriate dilutions of the transformation mixture onto non-selective medium. Fig. 1 shows the results of a typical experiment. Both the number of transformants and the proportion of surviving cells without heat shock may vary by up to threefold between different such experiments, depending on the protoplast preparation. However, the overall response to heat shock is always the same, with transformation efficiency best around 48 °C, and the survival of the protoplasts beginning to decrease at about 46 °C. Transformation efficiencies after heat shock treatment at 48 °C vary from about 200 to 1000 transformants per μg DNA, depending on the protoplast preparation and on the plasmid used. This represents an increase of about 5–10-fold in the yield of transformants, and about 0.5–2.5% of the viable cells are competent for transformation.

In order to determine the delay of onset of competence we transformed the protoplasts at different times before and after heat shock treatment at 48 °C (Table 1). Heat shock had no effect if it was applied after the addition of the DNA. Improvement of competence was observed immediately after the heat shock and there was a further slight increase in the yield of transformants after 10 min. Heat-shocked cells were still competent after 20–30 min.

The efficiency of transformation after heat shock is significantly increased only if the treatment is applied before the addition of DNA. The optimal effect is obtained when survival
Improved transformation of P. anserina

Fig. 1. Effect of treatment at various temperatures before transformation on the number of transformants. Protoplasts were prepared as described in Methods, divided into 50 μl portions (5 x 10⁷ cells) in 1.5 ml microcentrifuge tubes and treated for 5 min at the temperatures indicated. The tubes were briefly cooled, and 1 μg DNA was added to each tube after 10 min incubation at room temperature. Then the samples were treated as for standard transformation. Viability of the cells after transformation was estimated by plating various dilutions onto non-selective medium. ---, Number of transformants; ----, plating efficiency.

Table 1. Effect of the time of addition of DNA to the heat-shocked protoplasts on the number of transformants

<table>
<thead>
<tr>
<th>Time of addition of DNA</th>
<th>No. of transformants per μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA without heat shock</td>
<td>Expt 1 80 Expt 2 116</td>
</tr>
<tr>
<td>DNA added:</td>
<td></td>
</tr>
<tr>
<td>10 min before heat shock</td>
<td>Expt 1 10 Expt 2 –</td>
</tr>
<tr>
<td>Just before heat shock</td>
<td>Expt 1 20 Expt 2 –</td>
</tr>
<tr>
<td>Just after heat shock</td>
<td>Expt 1 348 Expt 2 –</td>
</tr>
<tr>
<td>10 min after heat shock</td>
<td>Expt 1 418 Expt 2 –</td>
</tr>
<tr>
<td>20 min after heat shock</td>
<td>Expt 1 406 Expt 2 –</td>
</tr>
<tr>
<td>30 min after heat shock</td>
<td>Expt 1 – Expt 2 346</td>
</tr>
<tr>
<td>First heat shock at 44°C, 30 min at 20°C and DNA added 30 min after a second heat shock at 48°C</td>
<td>Expt 1 – Expt 2 116</td>
</tr>
</tbody>
</table>

---, Not tested.

of the cells begins to be altered. These results may explain why heat shock has not been used in fungal transformations. First, workers attempted the heat shock treatment at temperatures too low to be effective, and secondly the heat shock was applied after the addition of DNA. Our protocol can be attempted with other fungi (the optimal temperature may of course differ for different organisms).

The nature of the mechanism responsible for the improvement of competence after the heat shock treatment is unknown. As the results in Table 2 demonstrate, the improvement in transformation yield after heat shock does not depend on the selectable marker used for transformation, indicating that there is no relation with the expression of the transforming gene. The effect may be rather at the level of the physiological state of the protoplasts. Treated cells may have increased their capability to take up or (and) to integrate the transforming DNA.

The experiments described above were performed using 1 μg DNA (20 μg ml⁻¹) in each transformation assay. In other experiments, the number of transformants obtained increased
proportionally with the quantity of added DNA in the range 10–100 µg ml⁻¹. Addition of transformants can be obtained. This is sufficient to perform the direct isolation of genes from DNA (100 µg ml⁻¹) as carrier DNA increased the transformation efficiency by three- to four-fold, but only at low concentrations of the transforming vector. With or without carrier DNA the system appears to be saturated at the same level, suggesting that for high DNA concentration the limiting factor is the number of competent cells. Nevertheless protoplasts can be obtained in large amounts, and using 150–250 µg DNA ml⁻¹ in each transformation experiment, thousands of transformants can be obtained. This is sufficient to perform the direct isolation of genes from a P. anserina genomic library. Such a library has been realized in the cosmid pHC79 containing the ura5 gene. Cloning of some genomic sequences has now been undertaken.

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REFERENCES


