A Method for the Re-isolation of an Autonomously Replicating Plasmid from Aspergillus Transformants

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An autonomously replicating plasmid is expected to increase the frequency of Aspergillus transformation. To construct this type of plasmid, we developed a rapid method of re-isolating autonomously replicating plasmids from Aspergillus transformants. Transformants grown in MM medium under selective pressure for 1-2 days were converted to protoplasts with a cell wall lytic enzyme (e.g. Yatalase). The protoplasts were lysed with phenol/chloroform followed by precipitation with ethanol. The total DNA was treated with RNaseA, re-precipitated with PEG, and then used to transform E. coli. These re-isolated plasmids were mainly the plasmid monomer.

The advantages of an autonomously replicating plasmid include an increased frequency of Aspergillus transformation, which would be especially valuable in gene cloning by the re-isolation of plasmids that complement mutant alleles. This system would also be valuable in gene expression studies. In this paper, we describe a rapid method for the re-isolation of autonomously replicating plasmids from Aspergillus transformants.

A. oryzae IFO5240 was used as the wild-type strain. A recipient strain deficient in ornithine carbamoyltransferase was obtained by UV irradiation of conidia from the wild-type strain. This was used in the transformation experiments. This mutant strain formed white conidia and could grow on minimal medium (MM) with citrulline or arginine, but not with ornithine. A. niger van Tieghem ATCC20739 (argB) was also used as a standard strain. Escherichia coli HB101 was used for plasmid preparation and for rescue of plasmids from A. oryzae and A. niger. The media for the growth of Aspergillus transformants were complete medium (CM) composed of dextrose-peptide (DP), and minimal medium (MM) composed of Czapek-Dox (CD), as described previously. Both ARpl (11.5 kb) and pDHG25 (10.5 kb) could be autonomously maintained in Aspergillus (AMA1) and carry the A. nidulans argB gene. These plasmids were obtained from Dr. A. J. Clutterbuck (University of Glasgow, UK). The plasmid BluescriptII SK− (Stratagene) was used for DNA manipulations. Plasmid pDG3 (7.8 kb) carrying argB gene from A. nidulans was cloned from strain ATCC53006. Plasmid pBXb2 (6 kb) was constructed by inserting the 3-kb XbaI fragment carrying argB gene from pDG3 into BluescriptII SK to form an integrative vector. Transformation of Aspergillus was based on the method of Gomi et al. with some modifications. After being washed with sterilized water the mycelia were suspended in a cell wall lytic solution composed of 20 mM phosphate buffer (pH 6.0), 0.8 M NaCl or 1 M (NH4)2SO4, as an osmotic stabilizer, and 0.5-2.0% Yatalase (Takara). After incubation with gentle shaking at 30°C for 3 h, the residual mycelia were removed by filtration through a 30-μm nylon mesh.

We compared the transformation efficiencies of A. niger to those of A. oryzae with the autonomously replicating plasmids, ARpl and pDHG25, and the integrative plasmid pBXb2 (Table 1). The AMA1 regions in the ARpl and pDHG25 plasmids were 6.1 and 5.1 kb, respectively. There was no significant difference in transformation frequencies using different DNA batches of each plasmid when transforming either Aspergillus strain. The plasmid ARpl transformed A. niger at a frequency of 730 transformants per microgram of DNA. This compared with 4 transformants per μg DNA using the integrative pBXb2: an approx. 190-fold increase in transformation efficiency. Similarly, ARpl transformed A. oryzae at a frequency of 93 transformants per μg DNA, compared to 0.7 transformants per μg DNA using pBXb2: an approx. 130-fold increase. The transformation efficiencies for A. niger were over 5-8-fold greater than those observed with A. oryzae for both the AMA1 plasmids and the integrative plasmid. These AMA1 plasmids would be especially valuable in shotgun cloning experiments for the isolation of useful genes from Aspergillus. The transformation frequencies of A. niger and A. oryzae with the integrative plasmid were approximately the same as those observed in the transformation experiment with A. niger and A. oryzae using a plasmid carrying the A. nidulans argB gene.

Total Aspergillus DNA was prepared by the following extraction method. Aspergillus transformants were cultivated on 10 ml of medium in 50-ml Falcon tubes at 30°C with gentle shaking. Aspergillus transformants were prepared by the method of Imura et al. with some modifications, using 0.5% Yatalase as a protoplasting enzyme. Cultivated transformants were mixed with 10 ml of filter-sterilized protoplasting solution composed of two-fold concentration and incubated at 30°C for 3 h with gentle shaking. The resultant protoplasts were collected by centrifugation (700 × g, 5 min), lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM EDTA and 0.5% SDS, followed by incubation at 37°C for 30 min, and then phenol/chloroform extraction was done. The resulting aqueous solution was treated by the conventional method. The DNA was precipitated with ethanol, treated with RNaseA, and then re-precipitated with PEG.

To discover to what degree the AMA1 fragment exists as a free plasmid in the Aspergillus transformants, total DNAs were extracted from transformants (three subcultures) cultivated in either MM or CM media for 1-3 days. The DNA extracted from the ARpl transformants of A. oryzae was electrophoresed on an agarose gel and the ARpl plasmid was located. The hybridization was done by the methods described previously. Radiolabelled argB (1.7 kb) was not hybridized to uncut chromosomal DNA of every transformants, but hybridized to the total DNAs prepared from transformants cultivated in CD medium at the position corresponding in size to the supercoiled ARpl monomer. On the

Table 1. Comparison of Transformation Efficiencies with Different Vectors

<table>
<thead>
<tr>
<th>Type</th>
<th>Vector</th>
<th>AMA1 length (kb)</th>
<th>A. niger transformants</th>
<th>A. oryzae transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA1 plasmid</td>
<td>ARpl</td>
<td>6.1</td>
<td>750</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>pDHG25</td>
<td>5.1</td>
<td>584</td>
<td>122</td>
</tr>
<tr>
<td>Integration</td>
<td>pBXb2</td>
<td>—</td>
<td>4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Transformation was done using the protoplast-forming method.
other hand, the same probe was weakly and mainly hybridized to those prepared from transformants cultivated in DP medium at the position corresponding in size to the supercoiled AR plasmid dimer or nicked monomer. These results were also observed with the AR plasmid transformants of A. niger. Similar results were reported by Gems et al., where AR plasmid was maintained in the free form and was not integrated into the Aspergillus chromosome.³³

To calculate the ratio of supercoiled monomer to total DNA, we analyzed the intensity of bands by densitometry (Ultrascan XL Enhanced Laser Densitometer, LKB). It was found that the proportion of cccDNA monomer to A. oryzae transformant total DNA was increased 2–25-fold by growth on MM medium with selective pressure. Similar results were obtained from A. niger transformants. Therefore, AR plasmid could be recovered efficiently from Aspergillus transformants by cultivation on CD medium for 1–2 days.

Table II. Comparison of Types of AR plasmid Rescued from Aspergillus Transformants

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>CD</th>
<th></th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monomer</td>
<td>Dimer</td>
<td>Total</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

Aspergillus transformants were cultivated in CD and DP media for 1–3 days at 30°C with gentle shaking. Aspergillus total DNAs were used to transform E. coli HB101. Three transformation experiments were totaled.

To prove that efficient recovery of the AR plasmid monomer plasmid from A. niger and A. oryzae transformants, the total DNA (1 μg) was used to transform E. coli HB101. The types of AR plasmid of the resultant ampicillin-resistant E. coli transformants were examined by growing Aspergillus transformants under different conditions (Table II). It was found that the AR plasmid plasmids were mainly rescued from Aspergillus transformants when cultivated in CD medium for 1–2 days.

This work will be useful in the development of cloning methods for the molecular genetics and molecular breeding of Aspergillus.

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References