Transformation in *Bacillus amyloliquefaciens*

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Received for publication 18 September 1970

The methodology and some of the requirements for the deoxyribonucleic acid-mediated transformation of an arginine auxotroph of *Bacillus amyloliquefaciens* to prototrophy are described.

*Bacillus amyloliquefaciens* is used for the commercial production of two exoenzymes, α-amylase (EC 3.2.1.1) and alkaline protease. In the past, many investigators have confused this organism with *B. subtilis*. However, previous studies (7) from this laboratory [employing deoxyribonucleic acid (DNA) hybridization, transduction, transformation, and biochemical data] have clearly shown that *B. amyloliquefaciens* is not biochemically or genetically related to *B. subtilis*. In this report, we describe the methodology and some of the requirements for the DNA-mediated transformation of an arginine auxotroph of *B. amyloliquefaciens* to prototrophy.

MATERIALS AND METHODS

The strains of *B. amyloliquefaciens* and *B. subtilis* employed were those described by Welker and Campbell (7). Strains N or N-10T of *B. amyloliquefaciens* were used as the source of wild-type donor DNA in most of the transformation experiments to be described. The arginine auxotroph (strain N-10) of *B. amyloliquefaciens* was used as the recipient strain. This strain was isolated by the multiple ultraviolet-irradiation technique described by Welker and Campbell (7). Spontaneous reversion to prototrophy has not been observed with this strain during 5 years in culture.

The liquid media used were Penassay Broth (Difco) and the minimal glucose medium of Spizizen (5) as modified by Bott and Wilson (2). The modified medium contained 0.072% instead of 0.02% MgSO₄. For some experiments, the modified glucose minimal medium was supplemented with 1-arginine (50 µg/ml) and either 0.02% acid-hydrolyzed casein (Nutritional Biochemicals Corp.) or 0.02% synthetic casein hydrolysate (a defined mixture of amino acids minus arginine based upon the levels of amino acids present in 0.02% casein hydrolysate). For plating media, 1.5% agar (Difco) was added to the appropriate liquid medium.

All growth experiments were carried out at 37 C in a New Brunswick gyrotory water-bath shaker (model G-76) at a speed of 133 rev/min, describing a 1.3-cm diameter circle.

For the isolation of DNA, cells were grown overnight in 25 ml of Penassay Broth (Difco) at 37 C. The cells were then inoculated into 500 ml of Penassay Broth and grown at 37 C for 3 to 4 hr (or until early stationary phase). The cells were removed by centrifugation and the DNA was isolated as described by Saito and Miura (4). The isolated DNA was stored in SSC (standard saline citrate; 0.15 m NaCl-0.015 m trisodium citrate, pH 7.0) at 5 C and was preserved with chloroform. Termination of the isolation procedure after the phenol step and dialysis of the resultant solution against several changes of SSC gave a product which was just as effective in transformation as that obtained by carrying out the entire procedure of Saito and Miura (4). The concentration of DNA was determined by the colorimetric diphenylamine method of Burton (3).

Cell growth for transformation was carried out as follows. *B. amyloliquefaciens* strain N-10 was grown overnight at 37 C on Tryptose Blood Agar Base (Difco). A portion of these cells was inoculated into Penassay Broth containing CaCl₂ (10⁻³ M). After 14 hr of growth at 37 C, the cells were removed by centrifugation and suspended in an equal volume of the modified glucose minimal medium. (All cell washes and suspensions of cells were done in modified glucose minimal medium.) The washed cells were added to the cell-growth transformation medium (minimal glucose medium plus 0.02% casein hydrolysate and 50 µg of L-arginine per ml) to give an optical density (OD) at 500 nm of 0.1 (approximately 2 × 10⁷ colony formers/ml) in a Bausch & Lomb Spectronic-20 colorimeter. Growth was followed by measuring the OD at 500 nm.

RESULTS AND DISCUSSION

Figure 1 is a growth curve of *B. amyloliquefaciens* strain N-10 in the minimal glucose medium supplemented with casein hydrolysate and arginine. During growth of the culture, cells were removed and assayed for competence by the transformation procedures described below.

The cells became competent (as indicated by the arrows) in the OD range of 0.60 to 0.85, which occurred after 7 hr of growth in this medium.

A combination of washed cells (0.9 ml) plus donor DNA (10 to 15 µg) was used for the transformation experiments. Controls consisted of 0.9 ml of washed cells plus 0.1 ml of glucose minimal medium (for a revertant count) and 0.9 ml of washed cells plus 0.1 ml of DNA previously in-

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Cubated at 37 C for 15 min with 100 µg of deoxyribonuclease (EC 3.1.4.5) in the presence of 1.2 mM MgSO₄. Transformation in the experimental tubes was terminated by the addition of deoxyribonuclease and 1.2 mM MgSO₄ followed by incubation at 37 C for 15 min.

Except as noted, all transformation tubes and controls were incubated at 37 C for 180 min in a gyrotory water-bath shaker. The cells were removed by centrifugation, suspended in an equal volume of glucose minimal medium, and 0.2 ml was plated on glucose minimal agar plates and glucose minimal agar plates containing synthetic casein hydrolysate minus arginine. The total number of viable cells was determined by plating on glucose minimal agar containing casein hydrolysate plus 50 µg of arginine per ml. Sterility controls on the DNA and other reagents were also included.

To determine the optimum time for transformation, experiments were conducted in which the time of addition of DNA and the transformation times were varied.

Table 1 shows the results of this experiment and indicates that the optimum time for the addition of DNA is 120 min and the optimum transformation time is 180 min. The total viable cell count did not increase during these longer incubation periods (being usually in the range of 1.5 x 10⁶ to 2.0 x 10⁶ cells/ml).

Figure 2 shows another experiment in which the time of addition of DNA was assessed by using the 180-min transformation time. These data are consistent with those presented in Table 1 and show that, after 120 min, the addition of DNA gives the maximum number of transformants when used in conjunction with a 180-min transformation time.

Table 1. Effect of time of addition of DNA and duration of transformation time upon transformation of B. amyloliquefaciens (N10; arg⁻) to prototrophy

<table>
<thead>
<tr>
<th>Time DNA added (min)</th>
<th>Transformants per ml at 60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>770</td>
<td>2,150</td>
<td>3,200</td>
</tr>
<tr>
<td>90</td>
<td>930</td>
<td>3,090</td>
<td>4,150</td>
</tr>
<tr>
<td>120</td>
<td>1,740</td>
<td>2,910</td>
<td>4,900</td>
</tr>
<tr>
<td>150</td>
<td>2,450</td>
<td>4,230</td>
<td>4,900</td>
</tr>
<tr>
<td>180</td>
<td>2,900</td>
<td>3,650</td>
<td>4,140</td>
</tr>
</tbody>
</table>

DNA was added to the cells in glucose minimal medium at 37 C at different time intervals, and transformation was allowed to proceed for 60, 120, or 180 min. Transformation was terminated by the addition of deoxyribonuclease, and the number of transformants was determined as described in the text.

Previous studies in our laboratory have revealed that the addition of NaCl to growth media markedly stimulates the growth of B. amyloliquefaciens. The effect of NaCl on the development of competence and transformation was therefore determined by incorporating different levels of NaCl into the cell-growth transformation medium (minimal glucose medium supplemented with casein hydrolysate and arginine).

Figure 3 shows the effect of NaCl on the number of transformants at two transformation times, 120 and 180 min. It may be seen that the op...
transformation of NaCl at either time is in the range of 0.6 to 0.7 M. We now routinely add 0.65 M NaCl to all transformation media to achieve maximum transformation.

Figure 4 shows representative data on the effect of DNA concentration on transformation. The response is linear from 0.5 μg/ml to the saturation plateau level of 12 μg/ml. This is similar to the results obtained in other transformation systems although the levels of DNA required to achieve transformation are somewhat higher than those observed with other species of the genus Bacillus (1, 5, 6).

Table 2 shows that the DNA from B. amyloliquefaciens strains F, K, N, N-10T, P, SB, T, and VA transform the arginine marker of strain N-10 to prototrophy with a low but reproducible frequency. The DNA species from B. subtilis strain 168W and W23 do not transform B. amyloliquefaciens N-10 (arg-).

In separate experiments, the DNA of B. subtilis behaved as heterologous DNA by inhibiting transformation in the B. amyloliquefaciens system. This is in keeping with our previous results (7) that showed the DNA of B. amyloliquefaciens behaving as heterologous DNA in the B. subtilis transformation system. Studies are now in progress to develop conditions which will give higher transformation frequencies. We are also examining transformation with other auxotrophic markers and α-amylase negative mutants of B. amyloliquefaciens.

ACKNOWLEDGMENT

This study was supported by grant GB-6758 from the National Science Foundation.
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