Plasmid-Mediated Transformation in Bacillus megaterium

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A transformation system was developed for Bacillus megaterium by using antibiotic resistance plasmid deoxyribonucleic acid molecules derived from Staphylococcus aureus and Bacillus cereus. Lysozyme-generated protoplasts of B. megaterium allowed uptake of plasmid deoxyribonucleic acid in the presence of polyethylene glycol. Transformants expressed the antibiotic resistance determinants present on the plasmid deoxyribonucleic acid, and reisolated plasmid deoxyribonucleic acid yielded restriction endonuclease digestion patterns identical to those of the donor deoxyribonucleic acid.

The study of the genetic organization of Bacillus megaterium has been hampered by the lack of a genetic exchange system. Conventional competence regimen procedures used for Bacillus subtilis have proven to be unsatisfactory for transformation work with B. megaterium, although chromosomal DNA from B. subtilis subsp. amylobacteriaceiens has been reported to transform B. megaterium subsp. roseus (7). Schaeffer et al. (12) have demonstrated genetic recombination in fused polyethylene glycol (PEG)-treated protoplasts of auxotrophic strains of B. subtilis; Fodor and Alfoldi (6) reported recombination in a similar system when strains of B. megaterium were used. Chang and Cohen (4) have also used PEG-treated protoplasts to enhance plasmid-mediated transformation in B. subtilis. This paper describes another PEG-protoplast-mediated transformation system, which allows plasmids from Staphylococcus aureus and Bacillus cereus to be introduced into and expressed in B. megaterium.

MATERIALS AND METHODS

Strains and plasmid DNA. The recipient strain in all transformation experiments was B. megaterium 216S, a derivative of B. megaterium strain 216 (10) which is resistant to 100 μg of streptomycin per ml and has been cured of the 4- and 30-megadalton plasmids by treatment with 1 μg of novobiocin per ml at 43°C. Competent recipient cells were prepared by a standard B. subtilis procedure (1), and 1-ml samples were mixed with an equal volume of 20% (vol/vol) glycerol and frozen at −70°C. Noncompetent recipient cells were grown in minimal salts supplemented with 0.05% yeast extract (Difco) and 0.4% glucose (3). At various stages of growth (Fig. 1), 1-ml samples of the cultures were removed, mixed with equal volumes of 20% glycerol as described above, and stored frozen at −70°C.

Three plasmid DNAs (pUB110, pBC16, and pSC194) were used as donor DNAs in these transformation studies. pUB110 and pBC16 plasmid DNAs were isolated from transformed strains of B. subtilis BR151 (obtained from K. Bernhard). pUB110 was originally derived from S. aureus, has a molecular weight of 3.0 × 106, and encodes kanamycin/neomycin resistance (8, 9). pBC16 was originally derived from B. cereus GP7, has a molecular weight of 2.8 × 106, and codes for tetracycline resistance (2). S. aureus plasmid pSC194 is a natural recombinant plasmid (11) with a molecular weight of 4.9 × 106 and encodes chloramphenicol and streptomycin resistances. This plasmid DNA was isolated from a transformed strain of B. subtilis 3G18 (obtained from L. Rutberg).

The plasmid DNA isolated from B. subtilis strains and used for transformation experiments was isolated by a sodium dodecyl sulfate lysis modification of the procedure previously described for B. megaterium (3). A 1% (wt/vol) solution of sodium dodecyl sulfate in TES buffer (3) was substituted for a 2% (wt/vol) solution of Sarkosyl NL30 (Geigy Chemical Co.). Plasmid DNA recovered from transformed B. megaterium strains was further purified by zonal centrifugation in 10 to 30% linear sucrose gradients for 10 h at 18,000 × g in a Beckman SW27 rotor. This procedure separated the transformed plasmid DNA from the endogenous B. megaterium plasmids contained in the recipient. Restriction endonuclease digestion was performed on this DNA.

Transformation system. The transformation procedure used was basically the lysozyme-PEG procedure described by Chang and Cohen (4) for B. subtilis. Modifications for the B. megaterium system included the following: (i) we used recipient cells stored frozen at −70°C, either as competent or noncompetent cells (see below); (ii) the DM-3 regeneration medium contained 0.25 M sodium succinate, either 1.0 or 1.5% agar (Difco), and 100 μg of neomycin per ml (required for selection of pUB110 transformants); (iii) lysozyme was used at a concentration of 100 μg/ml for 15 to 30 min; and (iv) 4 h was allowed for expression of antibiotic resistance before plating.

B. megaterium regenerates a cell wall and begins dividing and forming chains in SMMP medium (4) with a 30 to 50% efficiency in 15 h at 37°C, thus allowing for direct selection of transformants, if desired, on nutrient agar plates containing an appropriate...
ate antibiotic. Cell wall regeneration is much slower in SMMP medium than on DM-3 plates, however, and no growth was observed on nutrient agar or on nutrient agar containing an antibiotic when platings were made 2, 4, or 8 h after transformation. Direct selection onto DM-3 plates containing antibiotic is satisfactory when made 4 h after transformation, although the B. megaterium strain used as recipient (strain 2165) generates extremely mucoid colonies which tend to run together, making colony counting and replica plating difficult. A higher concentration of agar (1.5%) in the regeneration plates seems to help this problem.

Restriction endonuclease digestions and agarose gel electrophoresis. All digestions were performed in a 40-μl reaction mixture. Reaction buffers have been described elsewhere (3). All reactions were carried out for 2 h at 37°C. In the case of BglII-BamHI double digestions, the BglII reaction was incubated for 1 h, the reaction was stopped by heating to 60°C for 3 min, and then the BamHI enzyme was added to the restriction mixture. In the case of the EcoRI-BamHI double digestions, the EcoRI reaction was incubated for 1 h, the reaction was stopped by heating at 60°C for 3 min, and then BamHI was added. No salt adjustments were made, and the second incubation was allowed to continue for 1 h. All restriction enzymes were products of New England BioLabs, Beverly, Mass., or Bethesda Research Laboratories, Rockville, Md.

Agarose gel electrophoresis was performed as described previously (3), using 1.2% agarose gels (medium EEO agarose; Sigma Chemical Co.) in a Tris-borate buffer system. Gels were run at room temperature for 16 h at 25 V.

RESULTS

Requirements for transformation. A 35-ml culture of B. megaterium 2165 was grown under the competence-inducing conditions employed for B. subtilis (1). Samples (1 ml) of this culture containing approximately 10^9 cells per ml were mixed with an equal volume of 20% glycerol as described above and stored frozen at −70°C for subsequent use. Six samples were later thawed at 37°C, centrifuged at approximately 4,000 rpm for 10 min in a Sorvall top centrifuge, and suspended in 0.5 ml of SMMP medium (4). Each sample was exposed to a different plasmid-lysozyme-PEG treatment (Table 1). After the transformation procedure was completed (4), cells were shaken gently at 37°C for 4 h to allow for expression of antibiotic resistance and were then plated onto DM-3 plates to obtain viable count data and onto DM-3 plates containing 100 μg of neomycin per ml to select for the pUB110 transformants. Non-lysozyme-treated samples (no protoplasts) were plated onto nutrient agar containing 10 μg of neomycin per ml as controls (lysozyme-treated cells do not produce colonies on nutrient agar after only 4 h of regeneration). Table 1 shows that both lysozyme and PEG were essential for transformation to occur in this strain of B. megaterium.

Transformation was also examined in B. megaterium cells grown under conditions which would not lead to competence in B. subtilis. Recipient cells grown in a supplemented salts medium were sampled at varying points on the growth curve (Fig. 1), added to an equal volume of 20% glycerol, and frozen at −70°C. After 10 days samples were thawed at 37°C, centrifuged, suspended in 0.5 ml of SMMP medium, treated with lysozyme and PEG, and transformed with approximately 1 μg of pSC194 DNA (see above). Protoplasts were allowed to regenerate overnight at 37°C in SMMP medium with gentle shaking and were then plated at appropriate dilutions onto nutrient agar and nutrient agar containing both chloramphenicol (10 μg/ml) and streptomycin (10 μg/ml). Table 2 suggests that B. megaterium may be transformed throughout much of the late logarithmic portion of the growth curve, i.e. that recipients need not be in state of competence. Microscopic observations of the overnight cultures revealed that about 50% of the protoplasts regenerated to a bacillary form and that the vast majority of these cells appeared as single cells or doublets. The rather poor relationship between viable counts and absorbance was mainly due to protoplast loss in the centrifugation step after the PEG treatment.

Although frequencies of transformation can
Table 1. Conditions required for transformation

<table>
<thead>
<tr>
<th>Treatment(s)</th>
<th>Viable counts/ml on DM-3 (a)</th>
<th>No. of colonies/ml on DM-3 + neomycin (100 µg/ml)</th>
<th>No. of colonies/ml on nutrient agar + neomycin (10 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme*</td>
<td>pUB110*</td>
<td>PEG†</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>7 x 10^7</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>4 x 10^6</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>5 x 10^7</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>6 x 10^6</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>4 x 10^7</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>5 x 10^6</td>
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</tbody>
</table>

* For lysozyme treatment, 10 µl of a lysozyme stock solution (5 mg/ml) was used; treatment was for 15 to 30 min at 37°C. The preparation was then centrifuged for 5 min at 4,000 rpm at room temperature, washed once in SMMP medium, and suspended in 0.5 ml of SMMP medium.

† Cesium chloride-ethidium bromide-purified DNA at approximately 50 µg of DNA per ml in 0.5X TES (3). A 10-µl amount of this DNA per 0.5 ml of washed culture suspension was added.

‡ For PEG treatment, 1.5 ml of a 40% (wt/vol) PEG stock solution in 2X SMM buffer (4) was used; 1.5 ml of 2X SMM buffer was added to samples not treated with PEG. The PEG in SMM or SMM was added immediately after DNA was added, and the mixture was incubated for 5 min at 37°C. PEG was diluted with 5 ml of SMMP medium and centrifuged for 10 min at 4,000 rpm at room temperature. The pellet was resuspended in 1 ml of SMMP medium.

Average of four plates.

Average of two plates.

—, Not plated (protoplasts).

Table 2. Transformation of protoplasts derived from various stages of the growth curve

<table>
<thead>
<tr>
<th>Klett reading (no. 66 filter)</th>
<th>No. of viable colonies/ml on nutrient agar + Strept + Chl</th>
<th>No. of transformed colonies/ml on nutrient agar</th>
<th>Frequency of transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>4 x 10^7</td>
<td>30</td>
<td>7 x 10^-7</td>
</tr>
<tr>
<td>75</td>
<td>7 x 10^5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>4 x 10^7</td>
<td>760</td>
<td>2 x 10^-4</td>
</tr>
<tr>
<td>120</td>
<td>1 x 10^7</td>
<td>&gt;4,000</td>
<td>&gt;4 x 10^-4</td>
</tr>
<tr>
<td>130</td>
<td>5 x 10^7</td>
<td>1,280</td>
<td>2.5 x 10^-4</td>
</tr>
<tr>
<td>165</td>
<td>3 x 10^7</td>
<td>30</td>
<td>1 x 10^-5</td>
</tr>
</tbody>
</table>

* Average of four plates (0.1 ml of appropriate dilution per plate). Strept, Streptomycin; Chl, chloramphenicol.

be calculated, these values should be regarded as only approximations because of the necessary lengthy elapsed time before plating and the variable efficiency of regeneration. The effect of transformation on the ability to regenerate a cell wall and subsequently divide in liquid medium has not been tested. However, similar results have been observed when plasmid pUB110 was used as transforming DNA, as well as with independently prepared batches of recipient cells.

Recovery of plasmid DNA from transformants. In three independent experiments frozen competent recipient cells of B. megaterium 2165 were transformed with pSC194, pUB110, or pBC16 plasmid DNA. Cells were allowed to regenerate in 1 ml of SMMP medium for 18 h at 37°C and then plated onto nutrient agar plates containing 0.1 ml of the appropriate antibiotic per ml. After overnight growth at 37°C, suspected transformed clones were screened for the presence of transforming plasmid DNA on 0.8% agarose gels.

Clones were screened by picking an isolated colony with a sterile toothpick and suspending it in 0.5 ml of 10% sucrose in TES buffer containing 100 µg of lysozyme per ml in a 1.5-ml Eppendorf tube. Cells were incubated at 37°C for 30 to 45 min, 0.25 ml of 2% Sarkosyl in TES buffer was added, and the mixture was blended gently with a Vortex mixer for 10 s. Samples were incubated for an additional 30 min at 37°C and centrifuged at 12,000 x g for 15 min at 4°C. A 50-µl amount of the supernatant liquid was loaded onto an 0.8% agarose gel and electrophoresed for 3.5 h at 100 V. Clones were selected randomly for screening; all clones examined (pSC194 [23 of 23], pUB110 [5 of 5], pBC16 [4 of 4]), the newly acquired plasmid was present in the transformed clone and comigrated with the transforming plasmid DNA used.

Two transformed B. megaterium clones were chosen at random for further study; one clone contained plasmid pUB110, and the other contained pBC16. A 1-liter amount of each culture was grown in supplemented minimal medium, and the transforming plasmid DNA was isolated and fractionated as described above. The restriction endonuclease digestion patterns of the two transformed plasmids isolated from strains of B. megaterium were compared with the digestion patterns of the original transforming DNA obtained from B. subtilis (Fig. 2). The digestion patterns suggest that both plasmids pUB110 and
pBC16 may be isolated intact and ostensibly unaltered after transformation into \emph{B. megaterium}. In each case restriction enzyme digestion patterns agreed with previously published data (2, 8).

**DISCUSSION**

A plasmid-mediated transformation system has been developed for \emph{B. megaterium}. Lysozyme and PEG pretreatment of the cells appears to be essential to allow transformation to occur. Transformation in the \emph{B. subtilis} system is most often associated with a state of physiological competence. However, \emph{B. megaterium} 2165 cells grown according to several competence-producing protocols (1, 5) have never been conclusively shown to take up and express DNA (unpublished data). A comparative study of PEG-lysozyme-treated competent and noncompetent recipient cells suggests that transformation may occur throughout much of the late logarithmic portion of the growth curve of \emph{B. megaterium} and that it is not dependent on the specific growth conditions normally used to induce competence in \emph{B. subtilis}. The levels of transformation observed were significantly lower than those obtained by Chang and Cohen (4) in \emph{B. subtilis}. One possible explanation, that \emph{B. megaterium} has a restriction modification system which restricts the plasmid DNAs, has been examined. The transforming activities of pUB110 plasmid DNA derived from \emph{B. subtilis} and of the DNA recovered from \emph{B. megaterium} transformants were compared. No detectable difference in the transforming activities of the two preparations were observed when \emph{B. megaterium} was retransformed, suggesting that restriction is not operating in this system.

To date, uptake and expression of only covalent closed plasmid DNA have been demonstrated by this protoplasting procedure in \emph{B. megaterium}. It will be of interest to determine in subsequent studies whether nicked circular and linearized plasmid and/or chromosomal
DNA transforms in this system. Gryczan and Dubnau (9) have reported no detectable transformation when linearized plasmid DNA was used to transform competent B. subtilis cells, although Chang and Cohen (4) reported that linearized plasmid DNA readily transformed protoplasted B. subtilis. Conversely, chromosomal DNA readily transforms competent B. subtilis, whereas no detectable transformation was observed with chromosomal DNA when protoplasted B. subtilis was used (4). These observations suggest that transformation for plasmid DNA may well involve different physiological parameters than transformation for chromosomal determinants.

ACKNOWLEDGMENT
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LITERATURE CITED