Transformation of *Bacillus polymyxa* with Plasmid DNA

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Received 13 January 1989/Accepted 18 July 1989

A plasmid transformation system was developed for *Bacillus polymyxa* ATCC 12321 and derivatives of this strain. The method utilizes a penicillin-treated-cell technique to facilitate uptake of the plasmid DNA. Low-frequency transformation (10⁻⁶ per recipient cell) of plasmids pC194, pBD64, and pBC16 was accomplished with this method. Selection for the transformants was accomplished on both hypertonic and nonhypertonic selective media, with the highest rates of recovery occurring on a peptone-glucose-yeast extract medium containing 0.25 M sucrose. Several additional plasmids were shown to be capable of transferring their antibiotic resistance phenotypes to *B. polymyxa* through the use of a protoplast transformation procedure which allowed for a more efficient transfer of the plasmid DNA. However, cell walls could not be regenerated on the transformed protoplasts, and the transformants could not be subcultured from the original selective media.

Strains of *Bacillus polymyxa* can produce a number of useful and potentially useful compounds, including peptide antibiotics, proteases, and a wide variety of carbohydrate-utilizing enzymes, such as β-amylase, β-D-xylanase, pullulanase, glucose isomerase, and polygalacturonate lyase (12, 13, 35–37). This bacterium has also been extensively tested as a source of 2,3-butyleneglycol (23, 26, 38), which can be converted into the feedstock chemical 1,3-butadiene.

For studies on the genetic control and enhanced production of these compounds, it would be useful to have a system for the transfer of genetic information within this species. Numerous methods of plasmid transfer between and within various *Bacillus* spp. have been reported. These include transduction (20, 25, 34), spontaneous transfer by a conjugationlike process (15, 21, 32), competent-cell plasmid transformation procedures for *B. subtilis* (10, 11), protoplast transformation procedures (5, 7, 18, 19, 27, 28), and a procedure developed for *B. brevis* involving exposing the cells to alkaline Tris hydrochloride (39). However, no useful procedure for plasmid transfer has been reported for *B. polymyxa*. This study was initiated to develop such a procedure.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used in this study.

Plasmid isolation procedures. Plasmid DNA was isolated from strains of *B. subtilis* by the procedure of Gryczan et al. (17) and by a modification of the procedure of Birnboim and Doly (4). In the Birnboim-Doly procedure, the plasmid-containing strain was grown overnight on a Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) plate at 35°C. Three to five colonies from the plate were touched with an inoculating loop and used to inoculate 12.5 ml of PGY (1.5% proteose peptone no. 3 [Difco], 0.5% glucose, 0.5% yeast extract). After growth with shaking for approximately 2.5 h at 35°C (A₅₄₀ 0.25), 12.5 ml of PGY containing 0.5 M sucrose and 10 U of penicillin per ml was added and incubation was continued for an additional 2 h. The cells were collected by centrifugation for 15 min at 3,000 x g and washed once in 10 ml of SMMP (equal volumes of SMM and 2x PGY; SMM consisted of 0.5 M sucrose, 0.02 M maleate, and 0.02 M MgCl₂ [pH 6.5]) before being transformed with plasmid DNA by a modification of the procedure of Chang and Cohen (7). The cells were gently suspended in 0.5 to 1 ml of SMMP, and 0.5-ml portions of the suspension were mixed with 0.1 ml of a plasmid preparation dissolved in equal volumes of 2x SMM and TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]). A 1.5-ml portion of 40% polyethylene glycol 8000 in SMM was added, and the solution was gently mixed and kept for 2.5 min at 0°C, followed by a 5-min incubation period at 35°C. SMMP (5 ml) was added, and the protoplasts were pelleted by centrifugation at 3,000 x g for 15 min. The protoplasts were suspended in 1 ml of SMMP and incubated for 30 min at 30°C with gentle shaking. Appropriate dilutions were made in SMMP and plated on TSA, PGYA, PGYSA, or DM3 (7) regeneration medium. PGYA was PGY with 1.5% agar. PGYSA was PGYA with added sucrose (0.25 M) and bovine serum albumin (0.01%). DM3 medium contained 0.5 M sodium succinate (pH 7.3), 0.5% Casamino Acids (Difco), 0.5% yeast extract, 0.5% glucose, 0.35% K₂HPO₄, 0.15% KH₂PO₄, 0.02 M MgCl₂, 0.01% bovine serum albumin, and 1% agar. Appropriate selective antibiotics were added in the...
following concentrations to nonhypertonic media and PGYSA: 5 μg/ml for neomycin, kanamycin, erythromycin, and chloramphenicol; 15 μg/ml for tetracycline; and 35 μg/ml for streptomycin. For DM3 medium chloramphenicol and erythromycin were added to 10 μg/ml; tetracycline was added to 30 μg/ml; and kanamycin, neomycin, and streptomycin were added to 100 μg/ml. The plates were incubated at 35°C for 2 to 5 days.

**Protoplast formation and transformation of protoplasts.** *B. polymyxa* was protoplasted by using either mutanolysin or a combination of penicillin and lysozyme. In the penicillin-lysozyme procedure the cells were grown and treated with penicillin as described above, collected by centrifugation, and suspended in 2.5 ml of SMMT (equal volumes of SMM and 2X Trypsinase soy broth [BBL Microbiology Systems]). Lysozyme was added to 10 mg/ml, and the suspension was incubated for 1 h at 35°C. The protoplasts were collected by centrifugation for 15 min at 3,000 × g, gently suspended in 1 to 2.5 ml of SMMT, and transformed with plasmid DNA as described above. In the mutanolysin production 25 ml of Trypsinase soy broth was inoculated with an overnight growth of *B. polymyxa* and incubated for approximately 3.5 h at 35°C (A565 0.4). The cells were harvested by centrifugation at 10,000 × g for 10 min, and the pellet was gently suspended in 2.5 ml of SMMT. Mutanolysin was added to a concentration of 200 U/ml, and the suspension was incubated for 2 h at 35°C. The protoplasts were collected by centrifugation at 3,000 × g for 15 min, gently suspended in 1 to 2.5 ml of SMMT, and transformed with plasmid DNA as described above.

Strains of *B. subtilis* were protoplasted and transformed with plasmid DNA by the procedure of Chang and Cohen (7).

**Development of a nonmucoid strain of *B. polymyxa*.** A nonmucoid strain of *B. polymyxa* was developed for use in this study by mutagenesis with N-methyl-N’-nitro-N-nitrosoguanidine. *B. polymyxa* ATCC 12321 from an overnight growth on TSA was used to inoculate 100 ml of Trypsinase soy broth in a 300-ml nephometer flask to an A565 of 0.02. Cells were grown with shaking at 35°C until an A565 of 0.2 was reached. A 6.8-ml portion of the cell suspension was mixed with 3.2 ml of 0.25-mg/ml N-methyl-N’-nitro-N-nitrosoguanidine in 0.05 M sodium acetate (pH 5.5). The mixture was incubated without shaking for 30 min at 35°C before centrifugation at 3,000 × g for 8 min at room temperature. The pellet was washed once in 10 ml of 0.85% NaCl and suspended in 1 ml of minimal medium (26). Dilutions (in 0.85% NaCl) were plated on TSA with 0.25 M sucrose and incubated at 35°C for 24 to 48 h. Colonies showing a nonmucoid colony type on the plates were checked on minimal media for wild-type nutritional requirements.

**RESULTS**

Several methods of plasmid transfer were tested for use with *B. polymyxa* ATCC 12321 and *B. polymyxa* U121, a mutant strain displaying a nonmucoid phenotype when grown on sucrose-containing media. Modifications of procedures for spontaneous transfer of plasmids (15, 31), plasmid transformation of competent cells (9, 24, 33), and plasmid transformation of cells treated with alkaline Tris hydrochloride (39) were not successful with the *B. polymyxa* strains.

**Transformation of protoplasted cells of *B. polymyxa*.** Numerous attempts were also made to create a protoplast transformation system for *B. polymyxa*. Lysozyme was used in the prototype *B. subtilis* protoplast transformation procedure of Chang and Cohen (7) and in numerous other transformation procedures for *Bacillus* spp. However, lysozyme was ineffective when used with *B. polymyxa*, producing fewer than 5% protoplasts, as determined by microscopic analysis.

Two protoplasting procedures described in the Materials and Methods section were developed for use with *B. polymyxa*. The methods used either mutanolysin or a combination of penicillin and lysozyme. More than 90% protoplasts, as determined by microscopic analysis and growth on hypertonic (DM3) and nonhypertonic (TSA) media, were produced when the procedures were used with either strain ATCC 12321 or strain U121. Small colonies could be obtained by plating the protoplasted cultures on the hypertonic DM3 medium. However, cell walls could not be regenerated on the protoplasted cells, and the resulting colonies could not be transferred to any of several variations of hypertonic and nonhypertonic liquid or solid media, including the original DM3 medium. Numerous modifications to the regeneration media that have been utilized with other organisms were tried without success, including the use of different hypertonic agents, the use of agar overlays, the use of different incubation conditions, and the addition of cell wall precursors, autoclaved *B. polymyxa* cells and cell wall preparations, minerals, polyvinylpyrrolidone, gelatin, and nutritional growth factors (1, 2, 8, 14, 22, 29).

The apparent transfer of several plasmids carrying antibiotic resistance markers was obtained with *B. polymyxa* ATCC 12321 when we used either the mutanolysin or the penicillin-lysozyme protoplasting method with plasmid

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**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Plasmid (phenotype)</th>
<th>Plasmid size (kilobases)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. polymyxa</em> ATCC 12321</td>
<td>Wild type</td>
<td>pUB1110 (Km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>4.5</td>
<td>ATCC*</td>
</tr>
<tr>
<td><em>B. polymyxa</em> U121</td>
<td>Nonmucoid strain</td>
<td>pSA2100 (Cm&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>7.1</td>
<td>This study</td>
</tr>
<tr>
<td><em>B. subtilis</em> 168</td>
<td>trpC2</td>
<td>pBC16 (T&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>4.2</td>
<td>BGSC*</td>
</tr>
<tr>
<td><em>B. subtilis</em> BGSC 1E6</td>
<td>thr-5 trpC2</td>
<td>pCI94 (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>2.9</td>
<td>BGSC</td>
</tr>
<tr>
<td><em>B. subtilis</em> 1E8</td>
<td>trpC2</td>
<td>pCI94 (Em&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>3.6</td>
<td>BGSC</td>
</tr>
<tr>
<td><em>B. subtilis</em> BGSC 1E9</td>
<td>thr-5 trpC2</td>
<td>pBD64 (Cm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>4.8</td>
<td>BGSC</td>
</tr>
<tr>
<td><em>B. subtilis</em> BGSC 1E18</td>
<td>thr-5 trpC2</td>
<td>pSA0501 (Sm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>4.2</td>
<td>BGSC</td>
</tr>
<tr>
<td><em>B. subtilis</em> BGSC 1E22</td>
<td>thr-5 trpC2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* American Type Culture Collection, Rockville, Md.
* Bacillus Genetic Stock Center, Ohio State University, Columbus.
TABLE 2. Summary of plasmid transfer experiments involving *B. polymyxa* protoplasts

<table>
<thead>
<tr>
<th>Recipient strain*</th>
<th>Plasmid</th>
<th>Recipients**</th>
<th>Transformants***</th>
<th>No. of transformants/recipient cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. polymyxa</em></td>
<td>pC194</td>
<td>1.0 x 10^7</td>
<td>2.8 x 10^4</td>
<td>2.8 x 10^{-3}</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>pBD64</td>
<td>2.7 x 10^7</td>
<td>1.8 x 10^4</td>
<td>6.7 x 10^{-4}</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>pBC16</td>
<td>3.4 x 10^7</td>
<td>2.1 x 10^3</td>
<td>6.2 x 10^{-5}</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>pSA0501</td>
<td>2.9 x 10^7</td>
<td>2.0 x 10^3</td>
<td>6.9 x 10^{-5}</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>pSA2100</td>
<td>3.5 x 10^7</td>
<td>2.3 x 10^2</td>
<td>6.6 x 10^{-6}</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>pEI194</td>
<td>2.2 x 10^7</td>
<td>3.0 x 10^1</td>
<td>1.4 x 10^{-6}</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>pC221</td>
<td>2.0 x 10^7</td>
<td>2.0 x 10^1</td>
<td>1.0 x 10^{-6}</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>pBD64</td>
<td>4.6 x 10^8</td>
<td>6.3 x 10^2</td>
<td>1.4 x 10^{-3}</td>
</tr>
</tbody>
</table>

* B. *polymyxa* ATCC 12321 was prepared by either a mutanolysin or a penicillin-lysozyme protoplasting procedure. *B. subtilis* 168 was transformed by the procedure of Chang and Cohen (7).

** Determined by growth on DM3 medium.

*** Transformants were selected for on DM3 medium containing 10 μg of chloramphenicol per ml for pBD64, pC194, pC221, and pSA2100; 10 μg of erythromycin per ml for pEI194; 30 μg of tetracycline per ml for pBC16; and 100 μg of streptomycin per ml for pSA0501. Approximately 1 μg of plasmid DNA was used in all transformations. Controls run without plasmid DNA produced 4.0 x 10^3 CFU/ml on media selective for pSA0501. All transformants were protoplasted cells and could not be transferred from the original hypertonic selective media.

transformation procedures and selection on DM3 medium containing the appropriate selective antibiotic (Table 2). The small size of the transformant colonies and the impossibility of subculturing prevented further characterization or confirmation of plasmid transfer.

**Transformation of penicillin-treated cells.** The lack of a cell wall regeneration technique for use with the *B. polymyxa* protoplasts prompted the study of potential injured-cell transformation techniques, in which the cell wall of the organism is damaged but not removed. Microscopic observation of different treatments suggested the use of either lysozyme or penicillin alone instead of in combination as in the protoplasting technique. Treatments with either penicillin or lysozyme produced fewer than 5% protoplasts, as determined by microscopic analysis. Plasmid transformation of lysozyme-treated cells produced no transformant colonies on hypertonic or nonhypertonic media.

However, transformant colonies were obtained when penicillin-treated cells of *B. polymyxa* UI21 were exposed to DNA from plasmid pC194. Microscopic analysis of cells from the selective plates and transfers of the colonies to nonhypertonic selective media showed that the transformants were intact cells. We tested the effect of including divalent cations in the SMM buffer used during the plasmid uptake step of the transformation procedure. Transformations were done with *B. polymyxa* UI21 as the recipient for approximately 1 μg of plasmid pC194 DNA. Selection was done on PGYA plus 5 μg of chloramphenicol per ml. The number of transformants per recipient cell was based on 4.2 x 10^7 CFU/ml of recipient cells. The original formulation of SMM, which included 20 mM MgCl_2_, produced more transformant colonies (90 CFU/ml, or 3.8 x 10^{-4} recipient/cell) on PGYA selective plates than did SMM containing 20 mM CaCl_2_ instead (10 CFU/ml, or 4.2 x 10^{-7} recipient/cell).

Controls run without plasmid DNA produced no colonies on selective media. When neither MgCl_2_ nor CaCl_2_ was added no transformant colonies were obtained. Subsequent experiments were run with the original SMM formulation. We tested different formulations of selective media. Transformations were done with *B. polymyxa* UI21 as the recipient for approximately 1 μg of plasmid pC194 DNA. When PGYA, DM3, and TSA (all containing 5 μg of chloramphenicol per ml) were tested, 100, 25, and 10 CFU of transformants per ml (1.9 x 10^{-6}, 4.8 x 10^{-7}, and 1.9 x 10^{-7} transformants per recipient cell, respectively, based on 5.2 x 10^7 CFU/ml of recipient cells) were produced, respectively. When PGYSA and PGYA (both containing 5 μg of chloramphenicol per ml) were tested, 506 and 123 CFU of transformants per ml (4.2 x 10^{-7} and 1.0 x 10^{-6} transformants per recipient cell, respectively, based on 1.2 x 10^8 CFU/ml of recipient cells) were produced, respectively. Controls run without plasmid DNA produced no colonies on selective media.

Plasmid DNA bands could be detected in *B. polymyxa* UI21 cells that had been transformed with plasmid pC194 by the penicillin-treated-cell technique (Fig. 1). The plasmid DNA bands appeared to have the same molecular weight as plasmid pC194 DNA obtained from *B. subtilis* BGSC 1E22. Plasmids pBD64 and pBC16 could also be used to transform *B. polymyxa* UI21 by the penicillin-treated-cell technique (data not shown). No detectable transformants were obtained with pSA2100 or pEI194 DNA. The use of plasmid DNA in excess of 1 μg had no effect on the transformation results. Plasmid pC194 DNA collected from both *B. subtilis* and *B. polymyxa* could be used to transform *B. polymyxa*. Transformation results for wild-type *B. polymyxa* ATCC 12321 were similar to those for strain UI21.

All of the plasmids transferred to the *B. polymyxa* strains were relatively unstable. Plasmid-containing strains that were grown overnight on nonselective media could not be transferred back to selective media. Frequent transfers of the plasmid-containing strains on selective media were necessary for viability.

**FIG. 1.** Agarose gel (0.7% of modified Birnboim-Doly plasmid DNA preparations. The preparations were additionally treated with RNase A (1 μl of a 2-mg/ml solution; Sigma) and 3 U of HindIII restriction endonuclease for 1 h at 37°C. The preparations were from *B. subtilis* 168 (lane A), *B. subtilis* BGSC 1E17 (pC39) (lane B), and *B. polymyxa* UI21 before (lane C) and after (lane D) transformation with plasmid pC194. All samples were 50 μl. The arrow points to the 2.9-kilobase linear form of plasmid pC194. Molecular size markers in kilobases are given to the left of the gel. Transformation was by the penicillin-treated-cell procedure, with selection on DM3 medium plus 5 μg of chloramphenicol per ml.
DISCUSSION

This paper describes the transformation of *B. polymyxa* cells with plasmid DNA. A transformation system for *B. polymyxa* was developed by using a cell wall injury treatment with penicillin. This penicillin-treated-cell transformation procedure was used to transfer plasmid DNA to *B. polymyxa* strains, with selection on either hypertonic or nonhypertonic media. Cells transformed by this procedure could be subcultured and used as donor strains for further plasmid transformation procedures. Plasmids pC194 and pBD64 were transferred by this technique, with selection for chloramphenicol resistance, and plasmid pBC16 was transferred by this technique, with selection for tetracycline resistance. Plasmid pC194 was originally isolated from *Staphylococcus aureus* (11), and plasmid pBC16 was isolated from *B. cereus* (3). Plasmid pBD64 is a chimeric plasmid developed from the *S. aureus* plasmids pC194 and pUB110 (16).

Several additional plasmids originally isolated from *S. aureus* could apparently be transferred to *B. polymyxa* protoplasts. This procedure provided a higher transformation frequency than did the penicillin-injured-cell procedure, but cell walls could not be regenerated on the transformed cells and the colonies could not be subcultured. The complexity of the *B. polymyxa* cell wall (6, 30) and the highly efficient cell wall removal obtained with the mutanolysin and penicillin-lysozyme protoplasting procedures may help to explain the inability to develop a cell wall regeneration procedure. Regeneration of the cell wall may require the presence of some cell wall material, although purified *B. polymyxa* cell wall preparations had no effect on regeneration.

The penicillin-treated-cell transformation procedure provides a method for transferring plasmid DNA to *B. polymyxa* at a very low frequency (~10^{-6} transformants per recipient cell) and cannot be used effectively with selection techniques that produce spontaneous mutant colonies above this threshold. However, the technique should be useful in transferring to *B. polymyxa* plasmids that have been developed by cloning techniques in *Escherichia coli* or *B. subtilis*. The technique might also be useful in other species of gram-positive organisms that have been resistant to other plasmid transfer techniques. Somewhat more efficient protoplast transformation methods showed that several additional plasmids could be transferred to *B. polymyxa*. Additional protoplast regeneration studies may prove useful with this bacterium.

ACKNOWLEDGMENTS

This study was supported by U.S. Department of Agriculture Hatch grant ILLU-50-0315 and by a University of Illinois Graduate College dissertation research grant awarded to D.H.M.

LITERATURE CITED