Protoplast Transformation of *Bacillus licheniformis* MC14

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A protoplast transformation system has been developed for *Bacillus licheniformis* MC14. Optimum regeneration conditions were achieved by raising the incubation temperature of the regeneration plates to 46 °C. Regenerated transformed colonies could be isolated in 3 to 5 d under these conditions. Plasmids introduced by this method were stably maintained by *B. licheniformis* MC14 and could be recovered and used to transform *Bacillus subtilis*.

INTRODUCTION

Alkaline phosphatase (APase) has been well studied in both Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus subtilis* and *Bacillus licheniformis*) systems. This enzyme is produced and secreted from cells which are subjected to phosphate-limiting growth conditions. The study of APase therefore encompasses inquiry into such mechanisms as the regulation of stress responses, the mechanism of protein secretion, and interactions between various environmental response systems.

B. licheniformis MC14 has been extensively used in our laboratory to study APase. This strain, which synthesizes 15 times more APase activity than has been reported for other *Bacillus* species (Hulett & Campbell, 1971; Spencer *et al.*, 1981), is an excellent system for biochemical studies of this enzyme. It does not, however, have an established transformation system. Our first goal was to develop a reliable transformation system for *B. licheniformis* MC14 in order to probe the relationship between multiple structural APase genes and multiple gene product destinations through recombinant DNA methods (Hulett, 1987). We have achieved transformation of *B. licheniformis* MC14 using a modified version of the Chang & Cohen (1979) *B. subtilis* protoplast transformation protocol. Plasmid DNA can be recovered from transformed cells without apparent deletions or rearrangements.

METHODS

Bacterial strains and plasmids. *Bacillus licheniformis* strain MC14 (Hulett & Campbell, 1971) was used for all transformation procedures. Plasmid pBC16 carrying a Gram-positive tetracycline-resistance gene was used for transformation (Bernhard *et al.*, 1978).

Media. All media used for protoplast transformation procedures were as described by Chang & Cohen (1979). Penassay broth (per litre: 4 g Difco nutrient broth, 2.5 g peptone, 1.5 g yeast extract, 3.5 g NaCl, 1.32 g KH₂PO₄, 3.68 g K₂HPO₄) was supplemented with 0.1% (w/v) glucose. 2 × SMM medium consisted of 1 M-sucrose, 0.04 M-maleic acid and 0.04 M-MgCl₂ at pH 6.5. DM3 (for regeneration plates) contained the following sterile solutions: 200 ml 4% (w/v) agar, 500 ml 1 M-sodium succinate (pH 7.3), 100 ml 5% (w/v) Casamino acids, 50 ml 10% (w/v) yeast extract, 100 ml 3.5% (w/v) K₂HPO₄, 100 ml 1.5% KH₂PO₄, 25 ml 20% (w/v) glucose, 20 ml 1 M-MgCl₂ and 5 ml filter-sterilized 2% (w/v) bovine serum albumin. Mannitol-based regeneration medium was as determined by Gray & Chang (1981). A sorbitol-based regeneration medium was made by replacing mannitol with sorbitol in the preceding medium. LB medium was made according to Maniatis *et al.* (1982).

Abbreviation: APase, alkaline phosphatase.

Transformation. Protoplast transformation was performed according to the method of Chang & Cohen (1979) as follows. The modifications made by us are noted under Results and Discussion. A culture of *B. licheniformis* MC14 was grown overnight in 5 ml LB medium broth supplemented with glucose to 1% (w/v); then 1 ml of this culture was inoculated into 50 ml of $1 \times$ penassay broth and grown to an OD_{540} of about 0.5 with agitation at 37 °C. Cells from 40 ml of this culture were pelleted in a clinical centrifuge and resuspended in 4 ml SMMP (1 vol. $2 \times$ SMM added to 1 vol. $4 \times$ penassay broth). This culture was subdivided into two cultures of 2 ml. One culture (control) received no lysozyme and the second culture received 0.5 ml filter-sterilized lysozyme (20 mg ml^{-1}) in SMMP. Both cultures were shaken for about 1 h at room temperature and protoplast formation was determined microscopically. When no whole cells were observed, protoplasts were pelleted in the clinical centrifuge, washed twice with 2 ml SMMP and finally resuspended in 1 ml SMMP. Protoplasts were subdivided into 0.5 ml aliquots, one of which (control) received no additions while the other received 10–100 μg DNA. Cultures were treated with 1.5 ml 40% (w/v) polyethylene glycol (PEG) for 2 min at room temperature and diluted by adding 5 ml SMMP. The cultures were then pelleted in the clinical centrifuge, resuspended in 1 ml SMMP, diluted, and plated to DM3 (or other) regeneration medium. Regeneration plates were incubated at 46 °C for 3–5 d in loosely closed plastic bags to retard moisture loss.

DNA isolation. Chromosomal DNA was isolated according to the method of Schleif & Wensink (1981). Plasmid DNA was isolated from *B. licheniformis* transformants according to the method of Gryczan *et al.* (1978).

RESULTS AND DISCUSSION

Development of the protoplast regeneration method

Bacillus licheniformis MC14 could not be transformed by competent cell transformation systems used for *Bacillus subtilis*. Other transformation systems developed for various *Bacillus* species, including cell mating in liquid culture (Landman & Pepin, 1982), filter mating with *E. coli* (Van Randen & Venema, 1984), and direct plasmid transfer (Gonzalez & Carlton, 1982), were also unsuccessful.

Since these transformation systems were not available for transformation of *B. licheniformis* MC14 we investigated the use of a protoplast transformation system in this strain. We began with the protocol established by Chang & Cohen (1979) for the transformation of *B. subtilis*. Our initial attempt failed because we were unable to establish conditions under which the *B. licheniformis* MC14 protoplasts would regenerate. Instead, after about 48 h incubation at 37 °C on succinate-based regeneration plates, L-form colonies appeared. These consisted of protoplasts with little or no cell wall material. These L-form cells were able to grow and divide in the absence of a cell wall. The colonies of L-forms were very small ($< 1 \text{ mm}$), hard, and partially embedded in the agar. Individual cells appeared as phase-bright spheres or, very rarely, as a sphere with a small tail. The colonies could not be replica-plated or picked. Continued incubation at 37 °C failed to produce normal cells. When entire colonies were excised from the regeneration medium and transplanted to a mannitol- or sorbitol-based regeneration medium (Gray & Chang, 1981) normal cells were regenerated within 24–36 h at 37 °C. Direct plating on this mannitol- or sorbitol-based medium following transformation failed to produce any colony growth. Therefore, we needed to alter conditions to achieve regeneration of these L-form colonies.

Several variations of the succinate-based regeneration medium were tested. We have found that *B. licheniformis* MC14 grows well in high-salt conditions, requires citrate as a component in a chemically defined medium, and is enhanced in growth by DL-alanine. Each of these components was added to the succinate-based regeneration medium: plates were formulated containing 50 mM- or 100 mM-NaCl, 50 mM-sodium citrate, or 50 μg DL-alanine ml^{-1} . None of these additions accomplished the desired effect of stimulating regeneration of L-form colonies. In addition, mannitol or sorbitol (40 g l^{-1}) was added as a supplement to the succinate-based regeneration medium without apparent improvement of regeneration. Bourne & Dancer (1986) reported an improvement in regeneration of *B. licheniformis* protoplasts when gelatin was used as the sole gelling agent for regeneration plates. This did not, however, prove true for *B. licheniformis* MC14.

B. licheniformis MC14 is a facultative thermophile capable of growth at temperatures up to 60 °C. The normal culture conditions under which APase is assayed in liquid defined medium

include an incubation temperature of 50 °C, at which excellent growth occurs. Chen *et al.* (1986) achieved successful protoplast regeneration in the thermophilic species *Bacillus stearothermophilus* by incubating succinate-based regeneration plates at 60 °C. By analogy we elevated the incubation temperature for regeneration plates of *B. licheniformis*. At 37 °C, the normal incubation temperature for growth of *B. licheniformis* on plates, no L-form colonies could be seen on regeneration plates until 40 to 48 h of incubation. These L-form colonies failed to regenerate upon extended incubation for up to 5 d. Raising the incubation temperature to 42 °C resulted in the appearance of L-form colonies on the regeneration plates within 20 h. After 40–48 h at 42 °C about 1% of these L-form colonies had regenerated to normal bacilliform cells, and after 5 d about 10% of the L-form colonies had regenerated. A 46 °C incubation temperature for regeneration plates also resulted in the appearance of L-form colonies within 20 h but about 10% of these L-forms regenerated within 40–48 h. Incubation for 5 d at 46 °C resulted in regeneration of virtually 100% of the L-form colonies. These L-form colonies represented approximately 1 in 1000 of the protoplasted cell population. Higher temperatures were also tried (up to 50 °C; data not shown) but these did not yield any improvement in regeneration speed over 46 °C. Higher temperatures also have the additional disadvantage of encouraging the growth of spreading colonies, probably derived from unprotoplasted whole cells, which overgrew the plate and inhibit the growth of regenerants.

Protoplast transformation experiments

These relatively efficient regeneration conditions allowed us to perform transformation of *B. licheniformis* MC14. A plasmid capable of replication in *Bacillus* (pBC16; Bernhard *et al.*, 1978) carrying a tetracycline-resistance marker was selected for this transformation study because *B. licheniformis* MC14 is highly sensitive to tetracycline and it can be used in the regeneration medium for direct selection of transformants. We used 10 µg of intact plasmid pBC16 to transform *B. licheniformis* MC14 and isolated tetracycline-resistant colonies from each transformation by direct selection. Transformation efficiency using 10 µg of CsCl-purified pBC16 was calculated as follows: (no. of transformants)/(no. of viable protoplast regenerants) × 100. Every 10⁸ cells protoplasted yielded about 10⁵ L-form-colony-forming protoplasts and, of these, about 10² were transformants. This gave a transformation efficiency of 0.1%, which was consistent to ±0.05% for 27 transformations. Our data for transformants of pBC16 which were regenerated nonselectively and then picked to Tryptose Blood Agar (Difco) plates supplemented with antibiotic indicated that about 0.1% of the regenerated colonies were resistant to the antibiotic when 10 µg of DNA was used. These numbers are consistent with those obtained from direct selection. The transformants were confirmed as containing pBC16 by isolation of plasmid DNA by a quick-screen procedure (Gryczan *et al.*, 1978). Plasmid DNA was obtained from 5–10 transformants from each of 27 transformations. The pBC16 isolated from the transformants appeared to be identical to the parent plasmid, as judged by the *Eco*RI digestion pattern (two fragments, of 3.2 kb and 1.7 kb). For 12 of the isolates, the *Hind*III and *Pst*I digestion patterns were also confirmed as identical to that of pBC16. Plasmid DNA introduced into *B. licheniformis* MC14 by this method thus appears to be stably maintained and can be recovered without apparent deletions or rearrangements. Plasmid pBC16 DNA isolated from transformed *B. licheniformis* was used successfully to transform competent *B. subtilis*, further suggesting that the plasmid was unaltered. Five micrograms of pBC16 plasmid DNA isolated from *B. licheniformis* MC14 by the quick-screen procedure had the same transformation efficiency as 1 µg of CsCl-purified DNA from *B. subtilis* and yielded about 100 *B. subtilis* transformants per µg DNA.

We were unable to select for *Bacillus* plasmids carrying a chloramphenicol-resistance marker because our strain is chloramphenicol resistant. We were also unsuccessful in using a plasmid carrying a kanamycin-resistance marker because kanamycin selection cannot be performed under these conditions. Grosch & Wollweber (1982) reported that increasing the concentration of kanamycin in the succinate-based regeneration medium from 20 to 300 µg ml⁻¹ would effect the desired selection. We found, however, that concentrations up to 2 mg ml⁻¹ were ineffective and therefore abandoned using kanamycin in any direct selection of transformed protoplasts. It

Fig. 1. Effect of varying DNA concentration on transformation efficiency. Number of transformants ml^{-1} was calculated by multiplying the number of transformants by the dilution factor for each plate and standardizing the figure per ml of protoplasts. The total volume for each protoplast preparation before plating was 1 ml (see Methods).

is possible that under our incubation conditions (46°C) kanamycin is inactivated in this medium. In the case of the tetracycline and erythromycin (pFB9; Barany *et al.*, 1982, and data not shown) markers which we did use, we were able to select transformants for antibiotic resistance directly on the regeneration plates.

In order to maximize the efficiency of our transformation procedure, we examined the effect of varying the amount of pBC16 plasmid DNA used per transformation. This study was carried out under the conditions outlined above, regenerating protoplasts at 46°C on succinate-based plates supplemented with $10\ \mu\text{g}$ tetracycline ml^{-1} . The results are summarized in Fig. 1. The highest DNA concentration tested, $100\ \mu\text{g}\ \text{ml}^{-1}$, gave about 1% transformation efficiency.

We explored methods to try to reduce the concentration of DNA required to achieve efficient transformation. DeCastro-Costa & Landman (1977) suggested that an inhibitory protein present in protoplasts of *B. subtilis* retards the reversion of protoplasts to whole cells by disrupting cell wall formation. We reasoned that a nuclease might also be present in the *B. licheniformis* protoplast preparation, increasing the amount of DNA required for successful transformation. To test these possibilities, we treated the protoplasts for 30 min at room temperature with $10\ \text{mg}$ protease K ml^{-1} . This did not increase transformation efficiency. We also tested to determine if PEG pretreatment would further permeabilize the protoplasts to DNA, resulting in a more efficient uptake of DNA. Pretreating the protoplasts with PEG for 2 min, washing the protoplasts, and then treating them with PEG in the presence of DNA also did not increase the efficiency of transformation.

In summary, successful protoplast transformation requires both efficient regeneration of protoplasts and efficient utilization of DNA in the transformation process. We have found that the most limiting step in our transformation procedure was the regeneration of protoplasted cells, which occurs in two steps: formation of L-form colonies and regeneration of these L-form colonies to normal bacilliform cells. As can be seen in our calculations of transformation efficiency, there is a 1% survival rate in the protoplast population that establishes L-form colonies. Once L-forms are present, continued incubation at 46°C results in complete regeneration of virtually 100% of this population. The efficient utilization of DNA by the protoplasts undergoing transformation is accomplished by adding $100\ \mu\text{g}$ of DNA to the protoplasts in the presence of PEG, which results in about 1% of the regenerating L-forms being transformants. The conditions we have outlined here are very useful for the efficient and rapid isolation of transformants of *B. licheniformis* MC14 carrying plasmid DNA.

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