Transfection of Corynebacterium lilium Protoplasts

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A protoplast transfection system has been developed for a lysine-producing bacterium, Corynebacterium lilium, using the DNA of phage CL31. Phage CL31 is lytic and specific to C. lilium and has a genome of approximately 48 kb. The transfection procedure involves a polyethylene-glycol-mediated introduction of the DNA into lysozyme-treated cells and has a maximum efficiency of $3 \times 10^4$ transfectants per $\mu$g DNA.

INTRODUCTION

Transfection is the introduction of cells by the naked nucleic acid isolated from a virus and results in the production of complete virus. There has been interest in transfection as a means of investigating basic biological processes such as entry of DNA, competence of bacteria and recombination using well-studied organisms such as Escherichia coli and Bacillus subtilis, but there have been only a few reports on the transfection of amino-acid-producing bacteria (Momose et al., 1976; Yoshihama et al., 1985). As these micro-organisms are industrially important it could be useful to design such a genetic process. In this report we describe a transfection system for a lysine-producing bacterium, Corynebacterium lilium.

METHODS

Bacteria and phage. All bacterial strains were obtained from the American Type Culture Collection: Corynebacterium lilium ATCC 15990 was used except where otherwise specified. The virulent phage CL31 was from the Genetica collection.

Media and culture conditions. BHI medium (3.7%, w/v, Difco Brain Heart Infusion, 25 mM-Tris/HCl pH 7.5) was used for routine growth. For solid medium Bacto-agar was added to a final concentration of 1.3% (w/v). Hypertonic solid medium for protoplast regeneration was BHI medium supplemented with 10% (w/v) sodium succinate, 2% (w/v) polyvinylpyrrolidone K-90 (Fluka) and 20 mM-MgSO$_4$. Soft agar (Difco Brain Heart Infusion, 25 mM-Tris/HCl pH 7.5, 20 mM-MgSO$_4$, 10% (w/v) sodium succinate, 0.8% (w/v), Bacto-agar) was used as overlayer and maintained liquid at 60°C before use. Phage were stored in SM medium (5.8 g NaCl l$^{-1}$; 2 g MgSO$_4$ l$^{-1}$; 50 mM-Tris/HCl pH 7.5, 0.1 g gelatine l$^{-1}$).

All cultures were incubated at 34°C, and growth was measured as $\text{OD}_{540}$.

Isolation of phage DNA. Phage CL31 and its DNA were prepared according to the procedure of Maniatis et al. (1982) for phage $\lambda$.

Preparation and regeneration of protoplasts. A 0.5% inoculum from an overnight culture was incubated in a BHI hypertonic medium containing 10% sodium succinate, 20 mM-MgSO$_4$, 25 mM-Tris/HCl pH 7.5. At $\text{OD}_{540}$ 0.4, ampicillin was added (4 $\mu$g ml$^{-1}$). The culture was incubated for 1 h at 34°C and the cells were harvested by centrifugation at 8000 r.p.m. for 6 min at 4°C in an HB4 rotor. The cell pellet was resuspended in the same volume of the fresh hypertonic medium containing 0.5 $\mu$g ampicillin ml$^{-1}$ and 1 mg lysozyme ml$^{-1}$ and incubated at 34°C. The formation of protoplasts was monitored by phase-contrast microscopy, counting spherical cells. In addition, at various times, samples were plated on hypertonic agar to determine the regenerative population and on BHI agar to determine the osmotically resistant population. Colonies were counted after 3 to 10 d incubation at 34°C.

Transfection. A 500 $\mu$l sample of protoplasts was centrifuged in 1.5 ml Eppendorf tubes for 2 min at room temperature, using a microcentrifuge. The pellet was washed with the same volume of hypertonic growth medium.
Table 1. Sizes (kb) of fragments produced by the action of restriction endonucleases on DNA of phage CL31

| Digestion of DNA with restriction enzymes and gel electrophoresis. The conditions of digestion were as recommended by the suppliers. The digested samples were analysed by electrophoresis in 0.7% agarose gels, stained with ethidium bromide and photographed with long-wavelength UV light (Maniatis et al., 1982). Electron microscopy. Purified phage stocks were negatively stained with 1% (w/v) uranyl acetate. Samples were examined at 60 kV in a Siemens Elmiskop electron microscope. For observation of DNA a modification of the Kleinschmidt method was used (Stevens & Charret, 1974; Davis et al., 1971).

RESULTS

Properties of phage CL31

Phage CL31 replicated in Corynebacterium lilium ATCC 15990 and 21793. We could not obtain plaques on the following related bacteria: Corynebacterium glutamicum ATCC 21491 and 13287, Corynebacterium hydrocarboclastum ATCC 21131, Corynebacterium sp. ATCC 21857, Brevibacterium flavum ATCC 14067, 21127 and 21528, Brevibacterium divaricatum ATCC 14020 and 21792, Brevibacterium ammoniagenes ATCC 6872, Brevibacterium lactoflumen ATCC 21086, Brevibacterium helvolum ATCC 19390, Brevibacterium ketoglumaticum ATCC 15587 and Brevibacterium sp. ATCC 21860. Negatively stained preparations showed a hexahedral head (62 nm wide) and a tail 370 nm long and 15 nm wide. This phage is very similar to phage λ. CP31 DNA contains several cleavage sites for a variety of restriction enzymes: EcoRI, BamHI, ClaI, PvuI, PstI and HindIII produced from 9 to 18 fragments. The size of CL31 was estimated as 45.8-47.9 kb by the sum of the sizes of fragments produced by ClaI, PvuI and BamHI (Table 1). Electron microscopic observations showed that the DNA is a linear monomer.

Protoplast formation

After the addition of lysozyme to the cells, portions of the suspension were diluted and plated for regeneration on hypertonic BHI medium and on BHI medium. In both media, the number of c.f.u. decreased rapidly during the first 5 h of incubation and more slowly afterwards (Fig. 1). In BHI medium osmotically stable cells are scored. In hypertonic BHI media the colonies arise from osmotically stable cells and osmotically sensitive cells able to regenerate. This latter category may be calculated by the difference between the two curves. The number of osmotically sensitive cells able to regenerate was used as a basis for calculating the frequency of transfection.
Transfection of Corynebacterium protoplasts

Fig. 1. Kinetics of protoplast formation. After addition of lysozyme, cells were incubated at 34 °C, and samples were plated at various times on hypertonic BHI medium (○) and on BHI medium (●). Colonies were counted after 10 d incubation. The variation in colony counts between replicate experiments was less than 15%.

Under the phase-contrast microscope, spherical cells appeared progressively: after 5 h incubation 90% of the population was spherical. The spherical cells lysed under the microscope if they were suspended in BHI medium or if 0.05% SDS was added.

Pretreatment of the culture with a low concentration of ampicillin prior to lysozyme treatment was required to obtain a high proportion of protoplasts: lysozyme alone was inefficient. It seems that a sensitization of the cell wall by such ampicillin treatment was required.

Transfection

Protoplast suspensions were mixed with phage DNA (0.1 μg ml⁻¹) after various times of lysozyme treatment. Before addition of lysozyme there was no transfection. The number of transfectants increased during the process of protoplast formation and reached a maximum between 20 and 24 h (Fig. 2). The ratio of transfectants to regenerable cells rose progressively, reaching a constant value of 6 × 10⁻³ after 20 h. This value should be an overestimation of the transformation efficiency since phage may not require a regenerable cell to replicate. The ratio of transfectants to the number of cells before addition of lysozyme was 4 × 10⁻⁵ after 20 h protoplast-forming treatment. However, this figure is too low since a large proportion of the initial cell population is not regenerable and may not be able to support the replication of viral DNA. We did not observe a significant enhancement of transfection by addition of protamine sulphate to the transfection mixture as reported for E. coli protoplasts (Benzinger et al., 1971).

It is noteworthy that the best time to obtain transfectants was after 20 to 24 h incubation with lysozyme. This is long after the cell population had become osmotically sensitive (4 to 6 h). Thus osmotic sensitivity is required for transfection, but the protoplasts are not yet ready to adsorb and/or replicate phage DNA with maximum efficiency.

To determine the dose response of transfection we used increasing concentrations of phage DNA after 19 h protoplast-forming treatment. There was a linear relationship between DNA concentration and plaque-forming units below 0.08 μg DNA ml⁻¹. Saturation was obtained at 0.2 μg ml⁻¹ (Fig. 3).
Fig. 2. Development of transfection ability as a function of the duration of the protoplast-forming treatment. The results are expressed relative to the volume of the initial protoplast suspension. The counts varied by less than 15\% between replicate experiments.

Fig. 3. Dose response of transfection for protoplasts obtained after 19 h lysozyme treatment. The results are expressed relative to the volume of the suspension after polyethylene glycol was added. Reproducibility of results was as for Fig. 2.

**DISCUSSION**

In this report we have described a virulent phage, CL31, that replicates in *Corynebacterium lilium*. The DNA from this phage is able to transfect protoplast preparations of this bacterium. The basic procedure for preparing the protoplasts is similar to those developed for other bacteria such as *Bacillus subtilis* (Gabor & Hotchkiss, 1979), *Streptomyces* (Bibb et al., 1978) or *Brevibacterium flavum* (Kaneko & Sakaguchi, 1979) and requires a sensitization treatment by ampicillin before the lysozyme treatment. The maximum efficiency of transfection measured in relation to the number of cells before protoplast formation was low, but the efficiency was much higher (about $6 \times 10^{-3}$) if related to the number of regenerable cells. This is because in this strain the protoplast-forming protocol gave a relatively low proportion of regenerable protoplasts.

The optimal competence of the protoplast mixture was obtained long after the culture was observed to be fully converted to protoplasts by microscopic observation or by measuring the number of osmotically sensitive cells by plating on hypertonic and normal medium. Thus competence for DNA entry and/or replication occurs after about 20 h of lysozyme treatment. The linear relation we observed between transduction and DNA concentration is similar to results obtained for protoplast transformation in *E. coli* (Benzinger et al., 1971). It is also reminiscent of transfection of intact *Bacillus subtilis* cells by DNA phages of the same size as, or smaller than, phage CL31 (Trautner & Spatz, 1973).

A major aim in developing this transfection system was to find the conditions that allow DNA entry into protoplasts to be used for transformation experiments. Using similar conditions we have been able to transform a related bacterium, *Brevibacterium lactofermentum*, by the hybrid plasmid pSA77, a 10.8 kb vector resulting from the fusion of an *E. coli* plasmid carrying three antibiotic-resistance genes and a *Brevibacterium lactofermentum* cryptic plasmid (unpublished results). Independently the same approach has been used to transfect and transform other corynebacteria (Katsumata et al., 1984).

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REFERENCES


