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Transformation of *Clostridium acetobutylicum* Protoplasts with Bacteriophage DNA

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Techniques for the transformation of *Clostridium acetobutylicum* protoplasts with bacteriophage DNA are described. Transformation required regeneration of protoplasts and a 2-h eclipse period.

Anaerobic bacteria are likely to play an increasing role in the developing field of biotechnology. The potential of anaerobes would be greatly enhanced with the development of genetic transfer systems, allowing the use of genetic manipulation techniques. *Clostridium acetobutylicum* is an anaerobe which is already used for the industrial production of acetone and butanol from molasses (13), and it has the advantage of being able to utilize a wide range of relatively cheap substrates, including pentose sugars (3, 6, 7, 9). As an initial step in the development of genetic transfer systems in *C. acetobutylicum*, we described techniques and media for the production and regeneration of stable protoplasts (2). Since attempts to transfer *Bacillus subtilis*, *Staphylococcus aureus*, and *Clostridium perfringens* plasmids to *C. acetobutylicum* protoplasts have been unsuccessful to date (unpublished results), we have investigated transformation of protoplasts with bacteriophage DNA.

C. acetobutylicum P262J, utilized in these studies, was derived from strain P262, which has been described previously (2, 3, 4, 14). This strain was originally isolated as an indicator for the assay of cell-free autolysin, as it exhibited increased sensitivity to the autolysin. In addition, it was found to produce lower levels of extracellular nuclease activity and was also sensitive to the bacteriophage CA1. Strain P262J was grown in the buffered *Clostridium* basal medium (CBM) of O'Brien and Morris (10). Cultures were incubated at 37°C, and all manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma-Scientific, Marietta, Ohio). Protoplasts of strain P262J were produced from exponential-phase cells which had been preconditioned by growth in CBM containing glycine (0.4% [wt/vol]) as described previously (2).

Phage CA1 was isolated from an infected fermenter during a bacteriophage outbreak which occurred in February and March 1980 at an industrial fermentation plant belonging to National Chemical Products, Ltd., Germiston, South Africa. Phage CA1 was propagated on strain P262J in CBM and assayed by the double agar layer method (1). Concentration and purification of phage lysates were achieved by two cycles of differential centrifugation at 6,000 × *g* for 10 min and at 57,000 × *g* for 2 h. Electron micrographs of purified high-titer lysates showed that phage CA1 had an icosahedral head 40 to 42 nm in diameter and a short, complex tail 30 nm in length, with elaborate tail appendages. The phage was similar in appearance to phage HM2, isolated from *Clostridium saccharoperbutylacetonicum* in Japan by Ogata et al. (11). Phage CA1 has a latent period of 80 min, a rise period of 80 min, and a burst size of 170 to 200 PFU $bacterium^{-1}$ (Fig. 1). Phage DNA was extracted with 0.01 M Tris-hydrochloride buffer-saturated phenol (pH 7.6), followed by exhaustive dialysis in 10 mM Tris-1 mM EDTA (pH 7.6).

Transformation by phage DNA was investigated by adding phage DNA (70 to 100 $\mu g\ ml^{-1}$) to protoplasts (1 ml) in CBM containing 0.3 M sucrose, 25 mM $CaCl_2$, and 25 mM $MgCl_2$. The transformation mixture was incubated at 37°C for different time intervals before samples (0.1 ml) were plated on regeneration medium (RM) (2) and incubated for 48 h to allow regeneration of the protoplasts. The RM plates were then scraped, resuspended in phage buffer, and assayed for PFU. The phage buffer contained (milligrams per milliliter) 0.24 $MgSO_4$, 0.01 gelatin, 1.5 KH_2PO_4, 4.0 NaCl, 3.0 $NaHPO_4$, 5.0 K_2SO_4, and 0.01 $CaCl_2$. Controls included phage DNA alone, protoplasts incubated without DNA, and protoplasts incubated with DNA which had been pretreated with 5 $\mu g\ ml^{-1}$ of DNase for 30 min before addition to the protoplasts.

Protoplasts of *C. acetobutylicum* were transformed by phage CA1 DNA (Table 1). Pretreatment of the phage DNA with DNase prevented transformation, and protoplasts did not release

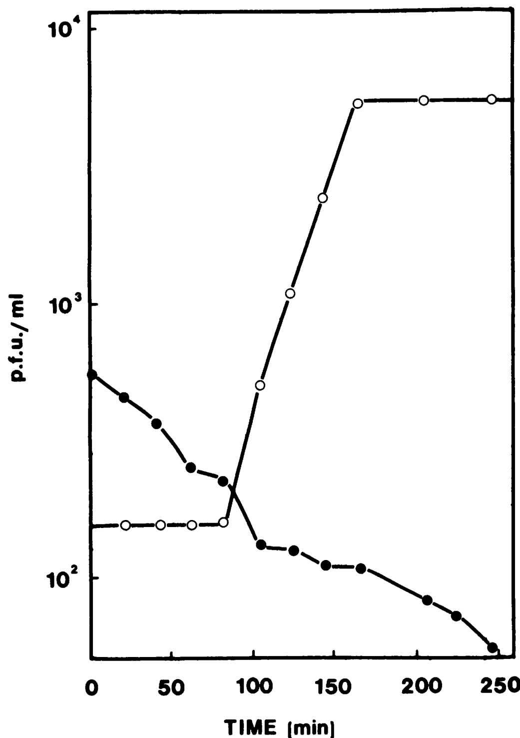

Fig. 1. Growth of phage CA1 on *C. acetobutylicum* cells and protoplasts. Phage CA1 was added to exponential-phase cells (○) and protoplasts (●). Samples were assayed for PFU at different time intervals.

mature phage spontaneously. Protoplast and DNA mixtures had to be incubated for 2 h at 37°C before regeneration of the protoplasts. Protoplasts infected with phage DNA never released mature phage particles, and they had to be regenerated on RM for 24 to 26 h before phage particles were observed. Plaques were not visible on the RM plates, and the regenerated protoplasts had to be scraped, resuspended in buffer, and assayed for PFU on CBM plates. It was therefore not possible to obtain the frequencies of protoplasts transformed, but our results indicate that phage CA1 DNA is taken up and expressed by *C. acetobutylicum* protoplasts which are allowed to regenerate.

The requirement for the 2-h incubation of protoplasts and DNA at 37°C is interesting, since it is unlikely to involve a slow DNA uptake mechanism. The rate of adsorption of phage CA1 to protoplasts and cells was determined. Although the rate of adsorption of phage to protoplasts was slower than the rate of adsorption to cells (adsorption constant of 2.6×10^{-6} ml^{-1} min^{-1} for protoplasts and 2.5×10^{-5} ml^{-1} min^{-1} for cells), the overall amount of adsorption is similar (80 and 86% phage adsorbed to

TABLE 1. Transformation of *C. acetobutylicum* P262J with phage CA1 DNA[a]

Expt	Incubation time (min)	Phage production[b]
DNA alone	0–180	–
DNA preincubated with DNase for 30 min before addition to protoplasts	0–180	–
Protoplasts only	0–180	–
DNA + protoplasts	30	–
DNA + protoplasts	60	–
DNA + protoplasts	120	+
DNA + protoplasts	180	+

[a] Purified phage DNA was added to protoplasts. The mixtures were incubated for different time intervals at 37°C before the protoplasts were regenerated on RM for 36 h and then assayed for phage production.

[b] –, No phage produced; +, phage produced.

protoplasts and cells, respectively, after 30 min). The necessity for the 2-h eclipse period is made more complex by the discovery that phage CA1 was unable to grow and was inactivated by *C. acetobutylicum* protoplasts (Fig. 1).

The inability to obtain PFU directly in transformation mixtures of competent *Agrobacterium tumefaciens* cells and phage DNA has been observed by Milani and Heberlein (8). However, when these same plates were scraped, resuspended in buffer, and replated, plaques were obtained. This was thought to be due to either slow replication of the phage or to a burst producing only a few phage particles. It may also reflect a requirement for growth or replication similar to that found in *B. subtilis* (12) and *Bacteroides thetaiotaomicron* (5).

The transformation system in *C. acetobutylicum* will be utilized in developing phage CA1 DNA as a possible vector for the transfer of recombinant DNA molecules into *C. acetobutylicum* cells.

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