APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1988, p. 264–267
0099-2240/88/010264-04$02.00/0

Vol. 54, No. 1

Transformation of *Clostridium perfringens* L Forms with Shuttle Plasmid DNA

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Received 13 August 1987/Accepted 10 October 1987

L-form (L-phase) cultures of *Clostridium perfringens* were tested for their transformability with plasmid DNA. Three L-form strains were transformable, but one, strain L-13, was superior to the others. This strain was easily and reproducibly transformed with previously described shuttle vectors which were derived from either *C. perfringens* or *Escherichia coli*. Strain L-13 was transformable by a variety of methods, and a new micromethod worked well under both aerobic and anaerobic conditions. The maximal number of transformants was attained after strain L-13 was exposed for 4 h to the transforming DNA and polyethylene glycol. Viable counts determined in tubes of semisolid brain heart infusion medium containing 10% sucrose, with or without 2 μg of tetracycline per ml, showed a transformation rate of 3.9×10^{-5} (transformants per viable cells).

Although plasmid DNA from *Clostridium perfringens* has been introduced by transformation into *Escherichia coli*, where it may (1, 2, 10, 12) or may not (3) be expressed, *C. perfringens* itself has been refractory to transformation with clostridial DNA. Since clostridial genes might best be studied by cloning into the homologous species, considerable interest centers around a transformation system for *C. perfringens* and plasmid vectors for cloning in this species. The creation of shuttle vector plasmids that can replicate and express tetracycline resistance in both *E. coli* and *C. perfringens* has been described by Squires et al. (12). These significant first reports of shuttle vectors and transformation (4) in *C. perfringens* confirm the unpublished results of others (D. E. Mahony, I. Roberts, and J. I. Rood, personal communication) showing that, unlike *E. coli*, the intact bacillus form of *C. perfringens* cannot be transformed easily. Heefner et al. (4) successfully transformed L-phase (L-form) variants of *C. perfringens* and described another wall-less form of the organism called autoplasts that could be transformed and would then revert to the bacillus form under appropriate conditions. These researchers and others (5, 8, 10) showed that reversion of stable L-form *C. perfringens*, referred to as does not occur.

We have had success with transforming L-form cultures using some of the shuttle plasmids described by Squires et al. (12) and Roberts et al. (10). In this paper, we relate some of our experience with clostridial L-form transformation. More specifically, we describe a micromethod for efficiently and reproducibly transforming an L form of *C. perfringens*, referred to as L-13.

MATERIALS AND METHODS

Bacterial cultures. *C. perfringens* strains 1, 2, 13, 28-1, and 63 were from our culture collection. They were grown at 37°C in cooked-meat medium (Difco Laboratories, Detroit, Mich.) and stored in this medium at room temperature. L-form cultures were produced by growing the strains in brain heart infusion broth (Difco) containing 10% sucrose (BHIS) and 2 U of penicillin G sodium per ml (Crystapen; Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom) as described previously (8). The L-form cultures tested for transformation were referred to as L-1, L-2, L-13, L-28-1, and L-63. Strain L-13 proved to be the most valuable for transformation. We have previously described this strain, referred to as "Le chien" (5, 6, 8). After stable L-form cultures were confirmed by serial passage through penicillin-free medium and by their protoplast appearance under phase-contrast microscopy, the organisms were transferred to semisolid thioglycolate-sucrose-agar medium (5) containing 3.7% brain heart infusion, 10% sucrose, 0.1% sodium thioglycolate, and 0.25% agar. After 24 h of growth at 37°C, these strains were kept as stock cultures at 4°C until required. Viability was retained for at least 1 month under these conditions, after which subcultures were made. The melted semisolid medium was cooled to 45°C before inoculation with L-form organisms. Cultures were incubated in an anaerobic glove box (Forma Scientific, Marietta, Ohio), although the medium could support growth under aerobic conditions when it was boiled for 10 min and cooled before inoculation. *E. coli* HB101 (9) and *C. perfringens* L-form strain L-13 were used to prepare the plasmid DNA used in these experiments.

Plasmid DNA. Plasmids pJU13, pJU16, pJU10, and pCW3 were a kind gift of C. Squires of Synergen, Boulder, Colo. The pJU plasmids were shuttle plasmids prepared from pBR322 DNA and *C. perfringens* DNA as described elsewhere (12). These plasmids contain tetracycline resistance genes derived from *C. perfringens* organisms.

Viable counts of L-form organisms. Viable counts of L-form organisms were made on BHIS agar plates containing 0.5% bovine serum albumin or by growth in tubes of semisolid agar as follows. Serial 10-fold dilutions of the culture were made in 0.9 ml of BHIS, and from these, 0.4 ml was transferred to 5 ml of molten semisolid BHIS agar (ssBHIS; BHIS plus 0.25% agar) at 45°C. The tubes were mixed by hand rotation to distribute the organisms and chilled to solidify the agar. Tetracycline (final concentration, 2.0 μg/ml) was added to make ssBHIS selective for transformants (ssBHIS-Tc). After overnight incubation, the colonies suspended in the medium could be counted. Tubes containing 10 to 20 colonies per tube were the easiest to count. All viable counts were done in duplicate.

Preparation of plasmid DNA and gel electrophoresis. Plasmid DNA (pJU16) was isolated from *C. perfringens* L-form

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cultures or from *E. coli* by a modification of the alkaline lysis method of Maniatis et al. (9). Most experiments used *C. perfringens*-derived DNA. The solution used to suspend the bacterial pellet incorporated 25% sucrose rather than 50 mM glucose for better osmotic stability. Agarose gel electrophoresis was performed in Tris borate-EDTA buffer as previously described (9). DNA restriction endonucleases were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) or International Biotechnologies, Inc., and were used as specified by the manufacturers.

Transformation experiments. Three transformation methods were designed. The first method was essentially that described by Heefner et al. (4) in which a 50-ml overnight L-form culture was centrifuged and the resulting pellet was suspended in 0.8 ml of our SMM buffer (0.5 M sucrose, 0.02 M maleic acid, 0.02 M $MgCl_2$ [pH 6.5]). DNA (1 to 5 μl) (about 1 μg) was added, followed by 1.5 ml of polyethylene glycol (PEG) 8000 (40% in 2× SMM). The mixture was gently shaken for 3 min, 15 ml of BHIS was added, and the suspension was centrifuged at 6,000 × *g* for 10 min at 10°C. The pellet was suspended in 50 ml of BHIS, incubated anaerobically overnight at 37°C (grow-out step), and centrifuged. The pellet was resuspended in 0.5 ml of BHIS, and 100-μl samples were plated onto BHIS agar containing 0.5% bovine serum albumin, with or without 2 μg of tetracycline per ml, and inoculated into ssBHIS and ssBHIS-Tc.

A minimethod similar to the one described above was devised for transformation. A 1-ml sample of a 5-h L-13 culture in BHIS was centrifuged in a Microfuge for 1 min. The pellet was suspended in 50 μl of SMM buffer, to which 5 μl of DNA (about 1 μg), 5 μl of 2× SMM buffer, and 100 μl of PEG in 2× SMM were added. The mixture was gently swirled for 3 to 5 min, and 1 ml of BHIS was added. The complete suspension was transferred to 10 ml of BHIS for anaerobic incubation overnight, the culture was centrifuged, and the pellet was resuspended in 0.5 ml of BHIS. This suspension was assayed for transformants as described above.

The final transformation method we describe here is a new micromethod. L-form cultures (10 ml) of *C. perfringens* were grown in BHIS at 37°C for 18 h. A 1-ml culture sample was centrifuged for 10 s in a Microfuge, the supernatant fluid was discarded, and the pellet was suspended in 1 ml of BHIS. A 200-μl sample of this suspension was placed in a flat-bottom well of a 96-well microtiter titration plate (Costar, Cambridge, Mass.). To this were added 5 μl (about 1 μg) of pJU16 plasmid DNA, 5 μl of 2× SMM buffer, and 5 μl of PEG in 2× SMM buffer. The mixture was gently stirred for a few seconds with a micropipette tip and then incubated for 4 h at 37°C in the anaerobic glove box. To detect transformation, the 200-μl culture was added to 5 ml of BHIS containing 2 μg of tetracycline per ml (BHIS-Tc) and incubated for 24 h. Growth with considerable gas production indicated transformed cells. Control cultures that did not receive the transforming DNA were similarly treated to ensure that there were no tetracycline-resistant L-form organisms in the population. The transformation frequency was obtained by comparing the viable count of transformants in ssBHIS-Tc with that of the total population in ssBHIS. The effect of DNA concentration on transformation was determined by adding increasing amounts of DNA to the L-form organisms in the experiments described above. Approximate DNA concentrations were determined with ethidium bromide-agarose plates as described by Silhavy et al. (11), with a range of salmon sperm DNA concentrations as standards.

RESULTS

Transformation of *C. perfringens* L forms. Using the method described by Heefner et al. (4), we were able to transform strains L-2 and L-13 with pJU16 DNA. Further studies with L-13 demonstrated that it was transformable with two other tetracycline resistance plasmids, pCW3 and pJU13. L-28-1 was more difficult to transform or to detect transformation in because of the slightly higher tolerance of the organism to tetracycline. The other strains tested were not transformable or were transformable at such a low frequency that transformation was undetectable. Agarose gel electrophoresis of restriction endonuclease digestions of pJU16 DNA and the plasmid DNA obtained from transformed L-2 and L-13 with *Eco*RI and *Sau*3A yielded identical restriction patterns, providing further evidence of transformation (data not shown). Although tetracycline-resistant L-form colonies grew on BHIS agar plates with 0.5% bovine serum albumin and 2 μg of tetracycline per ml, the number of transformants detectable in ssBHIS-Tc was as great as 1,000-fold higher. Thus, all viable counting of L-form cultures was done by using ssBHIS with or without tetracycline. Isolated colonies suspended in ssBHIS could be removed with a Pasteur pipette for subculturing, if desired. Because L-13 was the superior strain for transformation, all further experiments used this strain. By the minimethod of transformation, L-13 was successfully transformed with pJU16, pJU13, and pCW3 DNAs. Of four strains of L-form organisms tested with the micromethod, only strain L-13 was transformed. Control cultures which received no DNA did not grow in BHIS-Tc.

Transformation rate. Because of the simplicity of the micromethod, the following experiments were performed to better characterize the method. To determine the time required for the uptake of plasmid DNA and expression of antibiotic resistance genes, pJU16 DNA, SMM buffer, and PEG were added to 250-μl volumes of L-13 cultures in microtiter wells. The cultures were incubated anaerobically, and at 1-h intervals, 100-μl samples were removed and serially diluted in 0.9 ml of BHIS, and 0.4 ml of the dilution was transferred to duplicate tubes of ssBHIS-Tc for viable counts. Transformants expressed tetracycline resistance after 1 h of L-13 exposure to pJU16 DNA, and a peak of maximum transformation occurred by 4 h (Fig. 1).

To determine whether the rapid rise in the number of transformants that was observed within 4 h represented multiplication of a few transformed cells or, rather, a time for DNA uptake and gene expression, we performed the following experiment. Overnight cultures of L-13 cells and tetracycline-resistant L-13(pJU16) cells were centrifuged and suspended in fresh broth as described for the micromethod of transformation. A mixture of these cells was made in a ratio of 10^8 to 600 CFU/ml, respectively, and distributed to microtiter wells in 200-μl volumes. This culture was designed to mimic a mixture of tetracycline-sensitive and -resistant organisms that might be found in a transformation experiment. The mixture of cells was grown anaerobically and assayed for viable counts in ssBHIS-Tc as described for the transformation experiments. By observing the multiplication rate of the tetracycline-resistant organisms over 4 h, one should be able to conclude whether the rapid rise in the number of tetracycline-resistant L forms in the transformation experiment was actually due to multiplication of a few newly transformed cells. The small number of tetracycline-resistant organisms in the mixed culture did not increase during the 4 to 5 h of incubation (Fig. 1 and 2).

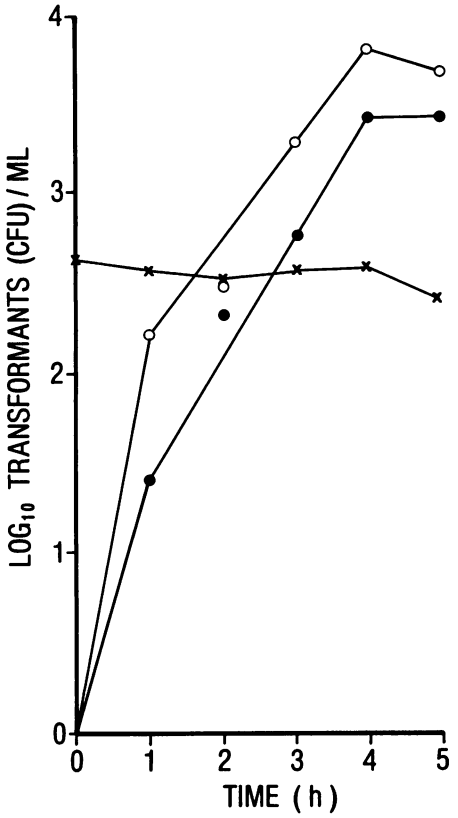

FIG. 1. Counts of L-form organisms transformed with pJU16. The micromethod was used to transform strain L-13 with about 1 μg of pJU16 DNA. Viable counts of L-form organisms were performed in ssBHIS-Tc medium at 1-h intervals after the addition of pJU16 DNA, PEG, and SMM buffer. Two experiments (●, ○) were performed several months apart to test the reproducibility of transformation. In a growth experiment (×) using a mixture of L-13 organisms (10^8 CFU/ml) and tetracycline-resistant L-13(pJU16) organisms (6×10^2 CFU/ml), no increase in the number of tetracycline-resistant L forms was observed in 5 h.

Transformation occurred at a similar rate whether DNA was added to L-form cells at time zero or after the cells had been kept for 4 h in the wells of the microtiter plates (Fig. 2). The transformation response of L-13 to increasing concentrations of pJU16 DNA is shown in Fig. 3, where a linear response to DNA concentrations from 0.019 to 0.075 μg is followed by a plateau up to 0.6 μg of DNA. The 1.2-μg concentration of DNA showed a threefold increase in transformants over the 0.075-μg concentration.

Transformation frequency. By performing the viable count assays in ssBHIS with and without tetracycline, an estimation of transformation frequency could be made. Results obtained by the method of Heefner et al. (4) gave a transformation frequency of 3.5×10^{-4} (transformants per total viable cells) after the grow-out period. The minimethod demonstrated a frequency of 4.4×10^{-4} after the grow-out period, and the micromethod demonstrated a frequency of 3.9×10^{-5}.

DISCUSSION

We have described different means of transforming *C. perfringens* L-form cultures of strain 13 (L-13) and have emphasized a micromethod which is both simple to perform

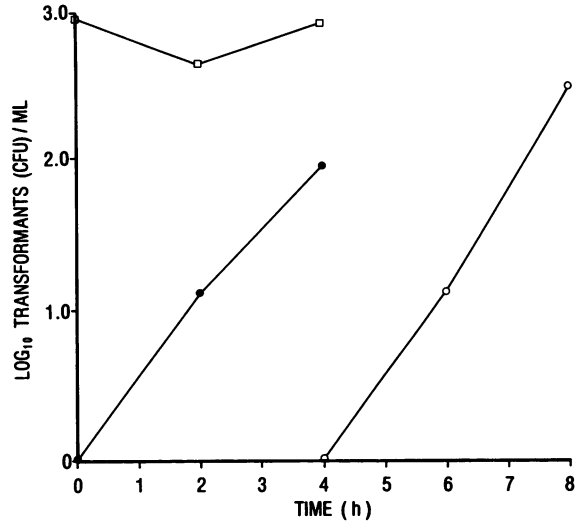

FIG. 2. Counts of L-form organisms transformed with pJU16. Volumes (200 μl each) of L-13 placed in wells of a microtiter plate received about 1 μg of pJU16 DNA, PEG, and SMM buffer at 0 (●) or 4 (○) h. Viable counts of L-form organisms were performed in ssBHIS-Tc over 4 h. A growth experiment (□) using a mixture of L-13 organisms (10^8 CFU/ml) and tetracycline-resistant L-13(pJU16) organisms (10^3 CFU/ml) demonstrated no increase in the number of tetracycline-resistant L forms in 4 h.

and reproducible. In addition, we described an L-form culture which proved useful for transformation. Unlike Heefner et al. (4), we were unable to transform autoplasts. Strain 2 produced a large number of protoplasts or autoplasts when it was incubated at room temperature in sucrose broth, but strain 13 did not. However, strain 13 protoplasts could be produced by lysozyme or penicillin treatment of the bacillus form of the organism, as well as by exposure to bacteriocins (7), which resulted in cell wall removal. Reversion to the bacillus form by either L-13 or L-2 was never observed. In the accompanying paper, Roberts et al. (10), using L-13 to demonstrate transformation and expression of a newly constructed shuttle vector, report similar results.

ssBHIS proved to be a sensitive means of growing L-form organisms, and viable counts were approximately 1,000-fold

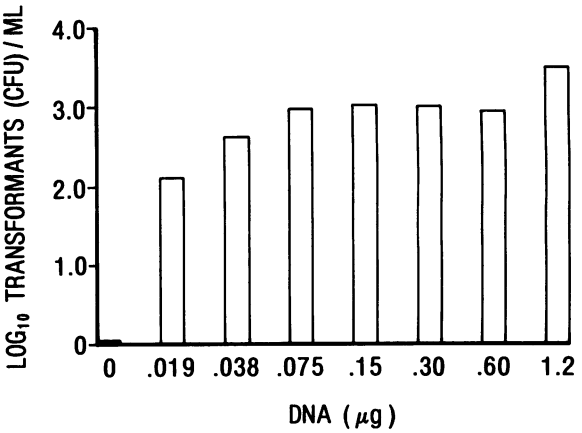

FIG. 3. Counts of L-form organisms transformed with different amounts of DNA. Increasing amounts of pJU16 DNA were added to L-13 organisms for the micromethod of transformation. After 4 h, viable counts of transformed cells in ssBHIS-Tc were determined.

higher than those attainable on agar plates designed for L-form growth. Regardless of which system was used to enumerate the L forms, the transformation frequency determined was similar. The isolated L-form colonies could easily be removed from the semisolid agar with a Pasteur pipette for further culturing. The use of ssBHIS-Tc permitted the detection of transformants which might be missed on agar plates. When we used the method of Heefner et al. (4), the grow-out stage was required to detect transformation. This stage apparently allowed the small number of transformants to grow to detectable numbers after several hours of incubation in fresh medium. In contrast, the micromethod did not require a grow-out stage, and L-form transformants could be detected by directly inoculating BHIS-Tc or ssBHIS-Tc. Although the DNA concentration was not known for some of the L-13 transformation experiments, there seemed to be little difference in the transformation efficiencies of plasmid DNAs derived from *E. coli* and transformed L-13.

The degree of transformation observed with increasing concentrations of DNA indicated that small amounts of DNA (as little as 19 ng) were sufficient to transform L-13. Concentrations of DNA greater than 75 ng did not enhance transformation until 1.2 μg of DNA was added (Fig. 3). This result suggested that the L-form organisms might be saturated with DNA; that only a small proportion of the L-form population was capable of undergoing transformation; or that two populations of L forms (4) might exist, one which is easily saturated with DNA and a second which requires a higher DNA concentration for successful transformation. Heefner et al. (4) reported a nonlinear relationship of DNA concentration to transformation.

In no experiment did L-form cultures not treated with DNA give rise to transformants in ssBHIS-Tc. PEG was required, and no transformants were found in its absence. Increasing the PEG volume from 5 to 50 μl did not increase the number of transformants observed with the micromethod, nor did the addition of carrier DNA (2.5 to 10 μg of salmon sperm) to the reaction mixture.

The rapid rise in the number of transformants within 4 h of DNA addition defined the incubation time necessary for maximum transformation in the micromethod. We questioned whether the rapid increase in the number of transformants represented growth of a few initially transformed cells, since other data (5) indicated that L-13 could not grow this quickly. We attempted to show, by a designed growth experiment in which a low number of tetracycline-resistant L-13 cells were mixed with a larger background population of tetracycline-sensitive L-13 cells, that the tetracycline-resistant cells could not multiply to the extent observed in transformation experiments in 4 h. Indeed, little or no multiplication of tetracycline-resistant L forms occurred in this time (Fig. 1 and 2). These preliminary data suggest that the conditions were not appropriate for L-form growth (there was no dilution of the stationary-phase L-form population into new medium to stimulate growth) and that the 4-h period was required more likely for DNA uptake or gene expression than for multiplication of a few transformed cells. The time of addition of DNA to the L-13 cells kept in the microtiter plate did not seem critical since transformation appeared to be equally efficient whether DNA was added at 0 or 4 h (Fig. 2).

We recently found that the micromethod of transformation works equally well whether performed under aerobic or anaerobic conditions. Good growth of L-13 occurred in BHIS that had been boiled for 10 min and cooled before inoculation from the stock culture. The microtiter plates containing L-13, DNA, and PEG could be incubated in an aerobic 37°C incubator, and a viable count of L forms in ssBHIS was comparable when the culture tubes were incubated aerobically. Thus, no anaerobic apparatus is required to perform these experiments. Nevertheless, we used anaerobic conditions to collect the data described in this report.

Using the micromethod, we recently tested a new shuttle vector, pHR106, described in the accompanying paper by Roberts et al. (10), and found that it transforms L-13 as efficiently as the pJU16 plasmid. Thus, we have described a useful L-form culture of *C. perfringens* for transformation and have defined a simple micromethod that provides a good yield of transformants. Whether other shuttle vectors will transform L-13 must be determined as such plasmids become available.

ACKNOWLEDGMENT

We acknowledge the support of the Medical Research Council of Canada (MA 6610).

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