Transformation of a Methylotrophic Bacterium, Methylobacterium extorquens, with a Broad-Host-Range Plasmid by Electroporation

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An efficient method for the transformation of the methylotrophic bacterium Methylobacterium extorquens NR-2 with a broad-host-range plasmid, pLA2917, by electroporation was examined. Transformants of M. extorquens NR-2 expressing resistance to kanamycin were obtained after electric pulse. These transformants were found to harbor a single plasmid which was electrophoretically identical and homologous to pLA2917 obtained from Escherichia coli. Several factors which determined the transformation efficiency were optimized, resulting in a transformation efficiency of up to $8 \times 10^5$ transformants per μg of plasmid DNA by 10 pulses at a field strength of 10 kV/cm and a pulse duration of 300 μs.

Methylotrophic bacteria able to grow on one-carbon compounds, such as methanol and monomethylamine, as sole carbon sources have received considerable attention because of their potential commercial applications, including the production of single-cell protein, amino acids, pyrroloquinoline quinone, and poly-β-hydroxybutyrate. The utility of methylotrophs should be increased by the use of recombinant DNA technology.

In genetic studies with methylotrophic bacteria as the host organisms, the conjugative transfer system has been used exclusively for introducing DNA molecules into the cells owing to the lack of an effective transformation system. However, this system is not only technologically cumbersome and time-consuming; it is also limited in the number and type of plasmids used because the participation of plasmids carrying the gene transfer function is essential.

The electroporation technique has been widely used to transfer DNA into mammalian cells (4), plant protoplasts (8), and yeast cells (11). Recently, this technique has also been applied to transforming a variety of prokaryotic cells with plasmid DNAs (5, 7, 16). However, electric transformation of methylotrophic bacteria has not yet been reported (6). In order to develop a transformation system for methylotrophic bacteria, we here used the electroporation technique with a pink-pigmented facultative methylotrophic bacterium, Methylobacterium extorquens NR-2, and a 21-kb broad-host-range plasmid, pLA2917, as a model test system. The optimal electric conditions for transformation efficiency are described in this article.

MATERIALS AND METHODS

Bacterial strains and plasmids. M. extorquens was formerly called Protaminobacter ruber. M. extorquens NR-2, used throughout this study, is the same strain as P. ruber ATCC 8457. Cells of M. extorquens NR-2 were grown aerobically at 30°C in an inorganic salt medium (18) containing 1% (vol/vol) methanol (MIS medium). Escherichia coli HB101 (3) was grown with shaking at 37°C in Luria-Bertani (LB) medium (15). Growth was monitored by measuring the OD$_{560}$ with a Spectronic 20 spectrophotometer (Shimadzu Co., Kyoto, Japan). For plating, the media were supplemented with 1.5% (wt/vol) agar. Antibiotics were added to the media at 50 μg/ml (kanamycin) and 15 μg/ml (tetracycline) for both E. coli and M. extorquens NR-2. Plasmids pLA2917 (21 kb; Km$^R$/Tc$^R$) (1) and pRK2013 (48 kb; Km$^R$, ColE1 replicon) (1) were maintained in E. coli HB101.

DNA manipulations. Plasmid DNAs were prepared from E. coli by the alkali-sodium dodecyl sulfate method of Birnboim and Doly (2). Plasmid DNA was isolated from M. extorquens NR-2 by the acetone-alkali lysis method of Kim and Lidstrom (12). Plasmid preparations were treated with RNase A (EC 3.1.27.5; Boehringer Mannheim Biochemicals [BMB], Indianapolis, Ind.). Restriction endonucleases (BMB) were used as recommended by the supplier. Plasmids and restricted DNA fragments were analyzed by 0.5% agarose gel electrophoresis. DNA fragments were recovered from the agarose gel with a DEAE-cellulose membrane (NA45; Schleicher & Schuell Inc., Keene, N.H.). Southern hybridization was carried out as described by Southern (19). Analysis of DNA transferred onto nitrocellulose membranes was performed with the ECL gene detection system (Amerham International, Amersham, United Kingdom).

Conjugative transfer. Conjugative transfer of pLA2917 into cells of M. extorquens NR-2 was carried out by a triple mating procedure with E. coli HB101(pLA2917) as a donor strain and E. coli HB101(pRK2013) as a helper strain, as described by Figurski et al. (9).

Electroporation apparatus. Electroporation experiments were performed with a somatic hybridizer (model SSH-2; Shimadzu Co., Kyoto, Japan). A square-shaped pulse up to 700 V can be delivered by this model. The maximum duration could be changed from 100 to 500 μs. The characteristics of the electric pulse generated by this apparatus are illustrated in Fig. 1. The electroporation chamber used was type SSH-C11, with parallel electrodes spaced at 0.5-mm intervals. With this chamber, a maximum field strength of 14 kV/cm is possible. The pulse repeat interval was fixed at 0.5 s.

Transformation of M. extorquens NR-2 by electroporation. All operations were carried out at 4°C unless otherwise specified. Cells of M. extorquens NR-2 grown in MIS medium to the middle logarithmic phase (1.4 × 10$^9$/ml) were harvested by centrifugation at 6,000 × g for 10 min and washed with electroporation buffer (10 mM Tris-HCl, 2 mM MgCl$_2$ - 6H$_2$O, 10% [wt/vol] sucrose [pH 7.5]). Cells were

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resuspended in the same buffer at a cell concentration of \(7.0 \times 10^{10}/\text{ml}\). The cell suspension and the solution of pLA2917 (70 \(\mu\text{g/ml}\)) were mixed at a ratio of 9:1 (vol/vol) in an Eppendorf tube. The mixture (10 \(\mu\text{l}\)) was then transferred into a space between the electrodes of chamber, where it was held for 3 min. After being subjected to electric pulse, a 5-\(\mu\text{l}\) aliquot of the mixture was transferred to an Eppendorf tube, and then 0.2 ml of MIS medium was added to the tube. The cell suspension was then incubated for 2 h at 30°C to allow expression of the antibiotic resistance genes of pLA2917 prior to plating on MIS plates containing kanamycin. Transformants of \(M. extorquens\) NR-2 displaying \(Km^R\) were scored on the selective plates after incubation for 4 to 5 days at 30°C.

RESULTS

Replication ability of pLA2917 in \(M. extorquens\) NR-2. Since it has not yet been demonstrated whether pLA2917 can replicate in \(M. extorquens\) NR-2, the replication ability of pLA2917 in this bacterium was firstly examined by a conjugative transfer experiment. \(M. extorquens\) NR-2 transconjugants expressing resistance to both kanamycin and tetracycline were obtained at a frequency of \(10^{-2}\) to \(10^{-1}\) per recipient cell. Plasmid DNAs prepared from several transconjugants were undistinguishable from pLA2917 obtained from \(E. coli\) in electrophoretic mobility on agarose gels and in restriction pattern, indicating that pLA2917 replicates in cells of \(M. extorquens\) NR-2.

Transformation of \(M. extorquens\) NR-2 with pLA2917 by electroporation. Because of the lack of information on the conditions required for electroporation of methylotrophs, including \(M. extorquens\) NR-2, we chose the following initial conditions: field strength, \(14\) kV/cm; pulse duration, \(300\) \(\mu\text{s}\); and number of pulses, 10. Under these conditions, colonies of \(M. extorquens\) NR-2 appeared on MIS plates containing kanamycin at a frequency of \(3.0 \times 10^2/\mu\text{g}\) of DNA. No colonies were obtained without the addition of plasmid DNA. Analysis by agarose gel electrophoresis revealed that plasmid DNA with the same molecular size as that of pLA2917 was harbored in the presumptive transformants. Furthermore, the plasmid DNA strongly hybridized with a 3.4-kb \(HindIII-Sall\) fragment of pLA2917 used as the probe in Southern blot analysis.

Effects of electric conditions on transformation efficiency. When the relationship between field strength and survival rate was examined at a fixed duration (300 \(\mu\text{s}\)) and number of pulses (10 pulses), the survival rate of intact cells of \(M. extorquens\) NR-2 decreased considerably with increasing field strength (Fig. 2). The figure also shows the effect of field strength on transformation efficiency. When the field strength was changed over a range from 0 to 14 kV/cm at a pulse duration of 300 \(\mu\text{s}\) and pulse number of 10, no transformant was obtained at field strengths below 5 kV/cm.

FIG. 2. Effect of field strength on cell survival of \(M. extorquens\) NR-2 (○) and transformation efficiency (●). The duration and number of pulses were 300 \(\mu\text{s}\) and 10, respectively.

A field strength of 10 kV/cm, which resulted in a survival rate of approximately 50%, gave maximum transformation efficiency, \(2.8 \times 10^7/\mu\text{g}\) of DNA. At a field strength of 14 kV/cm, the efficiency decreased to 36% of the maximum value (ca. \(1.0 \times 10^7/\mu\text{g}\) of DNA).

To examine the effect of pulse duration and number of pulses on transformation efficiency, these conditions were changed, ranging from 100 to 500 \(\mu\text{s}\) and from 0 to 10 times, respectively, at a fixed field strength of 10 kV/cm. Transformation efficiency was considerably affected by both of these parameters. The maximum efficiency of \(8.0 \times 10^3\) transformants per \(\mu\text{g}\) of DNA was obtained at a pulse duration of 300 \(\mu\text{s}\) and 10 pulses.

Dependence of transformation efficiency on amount of DNA. When the relationship between the amount of DNA used and the number of transformants obtained was examined, the yield of transformants increased progressively as the DNA amount increased from 0.7 to 70 ng. On the other hand, transformation efficiency decreased with increasing amounts of DNA, indicating that plasmid DNA was present in saturating amounts for the cell density used.

Effect of growth phase on transformation efficiency. As shown in Fig. 3, when cells grown to the early, middle, or late logarithmic growth phase were used for electroporation, transformation efficiencies ranging from \(6 \times 10^7\) to \(8 \times 10^7/\mu\text{g}\) of DNA were obtained. However, a significant decrease in transformation efficiency was observed when cells grown to the early or late stationary phase of growth were used. From 40 to 50% of cells at all growth phases survived electric pulsation (data not shown).

DISCUSSION

This work shows that electric pulse-mediated transformation can be used for a pink-pigmented methylotrophic bacterium, \(M. extorquens\) NR-2. Since a 21-kb broad-host-range...
plasmid, pLA2917, has been introduced into many gram-
negative bacteria, including some methylotrophs such as M.
extorquens AM1 (formerly Pseudomonas sp. strain AM1) and
Methylophilus organophilum, by conjugation (1); it was
expected that this plasmid would also be able to replicate in
M. extorquens NR-2. As the desired transconjugants could
be obtained in a triple mating experiment, we used electropo-
ration to transform intact cells of M. extorquens NR-2
with pLA2917. When M. extorquens NR-2 was subjected
to electric pulse in the presence of pLA2917, transformants
harboring a plasmid corresponding to this plasmid were
obtained, indicating that pLA2917 could be successfully
introduced into cells of M. extorquens NR-2. The transforma-
tion efficiency was greatly affected by the electric condi-
tions (field strength, pulse duration, and number of pulses).
This result indicates that they are important parameters for
electric breakdown of the cell membrane of M. extorquens
NR-2.

In general, high transformation efficiency by electropora-
tion has been obtained under electric conditions that result in
50 to 75% cell death (17). We obtained transformation
 efficiencies ranging from $2 \times 10^5$ to $8 \times 10^4$ transformants per
pg of DNA by using 10 pulses at a field strength of 10 kV/cm
and a pulse duration of 300 μs, which gave 50% cell survival
for M. extorquens NR-2. Two types of pulse shapes are
generated by the commercially available electroporation
apparatus. One is an exponential wave form, and the other
is a square wave form. With both types of pulse wave forms,
wide-ranging transformation efficiencies ($10^4$ to $10^6$ trans-
formants per pg of DNA) have been reported for different
bacterial strains and different plasmids (13, 14, 21). The
 electroporation apparatus (SS-2) used in this study gener-
ates a square wave pulse. Although it is difficult to directly
compare the transformation efficiencies in our and other
studies on electroporation, the transformation efficiency of $2 
\times 10^5$ to $8 \times 10^4$ transformants per pg of DNA obtained with
a 21-kb plasmid (pLA2917) in this study is high enough for
practical use in genetic manipulation.

Most methylotrophic strains do not possess a natural
transformation system and have not been reported to be
transformable by artificial methods, such as CaCl$_2$-heat
shock, with a few exceptions (10, 20). Even in these cases,
not only was the transformation frequency very low, but the
results could not be reproduced. For this reason, plasmid
DNA has been introduced into cells exclusively by conju-
agation. However, this technique is limited to special plasmids
which can provide a conjugation or mobilization function.
The electroporation method described here is therefore
particularly helpful for transforming methylotrophic bacteria
with plasmid DNA. The use of the electroporation procedure
in addition to the conjugation technique should facilitate the
 genetic manipulation of methylotrophic bacteria.

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