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Electroporation of Pink-Pigmented Methylotrophic Bacteria

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ABSTRACT

Electroporation conditions were determined for electroporating the broad-host-range plasmid pHX200V-47-ml (22.4 kb) into four pink-pigmented methylotrophic strains (*Methylophilus methylotrophus* AS1, *Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX, and *Pseudomonas* sp. M27). For these methylotrophs, a high electric-field strength (15 kV/cm) and high DNA concentration (18.75 ng/μL competent cells) were necessary to produce plasmid-containing cells. The addition of 30% polyethylene glycol (PEG) to the electrocompetent cells resulted in a five- to six-fold increase in electroporation efficiency in strains *M. methylotrophus* AS1, *M. extorquens* AM1, and *M. organophilum* XX, and also allowed successful electroporation of *Pseudomonas* sp. M27 (no electroporants were obtained without PEG). The maximum electroporation efficiencies for *M. methylotrophus* AS1, *M. extorquens* AM1, *M. organophilum* XX, and *Pseudomonas* sp. M27 were 1.31×10^3, 2.57×10^3, 5.25×10^3, and 1.2×10^4 electroporants/(μg DNA, respectively). This, along with our previous article *(1)*, are the first reports of a plasmid being successfully electroporated into these strains.

Index Entries: Electroporation; methylotrophs.

INTRODUCTION

Methylotrophic bacteria are those capable of growing on one-carbon compounds and have been studied extensively owing to their potential

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commercial value, including the production of single-cell protein, amino acids, pyrroloquinoline quinone, and poly-β-hydroxybutyrate *(2)*. The potential value of methylotrophic bacteria for the commercial production of eukaryotic peptides has also been shown through the expression of a synthetic human α1 interferon, chicken ovalbumin, and mouse dihydrofolate reductase in *Methylophilus methylotrophus (3,4)*. Mouse dihydrofolate reductase has also been expressed in *Methylomonas methylovora (4)*.

To study the genetics of methylotrophs, it is necessary to transfer genes efficiently; conjugation has been the primary means in which recombinant DNA has been introduced into methylotrophs *(5)* (e.g., the *Escherichia coli* pyruvate dehydrogenase gene has been conjugated into *Hyphomicrobium* X so that this facultative methylotroph can utilize pyruvate and succinate *[6]*). Natural transformation using linear chromosomal DNA has only been observed in two methylotrophs, *Methylobacterium organophilum* XX *(7)* and *Methylococcus capsulatus (8)*, and natural transformation of plasmid DNA (pRK290, 20 kb) has been reported for *M. organophilum* XX *(9)*. Application of a $CaCl_2$ cold-shock procedure to the autotrophic methylotroph *Paracoccus denitrificans* yielded transformants at frequencies of 3×10^{-4} per microgram DNA for plasmid RP1, but these results could not be reproduced *(6)*. In general, microgram-amounts of DNA and long contact times (on the order of hours) are necessary for transforming methylotrophs, which makes this a very inefficient DNA transfer system *(10)*.

Broad-host-range vectors used for cloning in methylotrophs have been derived from either RP4 or RSF1010 representing the IncP or IncQ incompatibility groups, respectively. The vectors derived from RP4 are approximately 20 kb *(11)* and cloned genes make them even larger. This may cause a depression of the transformation efficiency, as is seen in *E. coli* DH5α, where increasing plasmid size reduces the transformation efficiency *(12)*. Indigenous plasmids have been observed in many gram-negative methylotrophs *(13)*, but have not been used as cloning vectors.

Although methylotrophs may be transformed through RP4-mediated conjugal transfer, electroporation has the advantage of being quicker (fewer steps), and cloning vectors may be constructed that need not have the large conjugative and mobilization genes necessary for RP4-mediated transfer. It has been shown that the replication function of plasmid RP4 (50 kb) consists of a 400 bp origin of replication under control of a transacting *trfA** function located on a 1800 bp DNA fragment. A separate 760 bp region, *oriT* is the origin for plasmid transfer. These three regions are needed for conjugal transfer to occur *(11)*.

Electroporation is a successful method for transferring plasmid DNA into gram-negative bacteria; for example, the broad-host-range plasmid pLA2917 (21 kb) was electroporated into *Methylobacterium extorquens*

NR-2 using a field strength of 10 kV/cm, a pulse duration of 300 microseconds, and 10 pulses *(2)*, but the efficiency was not very high (8×10^3 electroporants/microgram of DNA). For comparison, *E. coli* may be electroporated with an efficiency as high as 5×10^7 electroporants/μg DNA *(14)* and the efficiencies can range from 9×10^2 up to 1.6×10^5 using plasmid pKT231 and a field strength of 6250 V/cm *(15)*. In other strains which may be transformed by electroporation, efficiencies may vary from 5×10^1 in *Enterobacter aerogenes* to 4×10^5 in *Erwinia carotovora (15)*. In general, authors have noted the potential of electroporation in methylotrophs but have not given specifics *(10)*, although Lidstrom et al. *(16)* mention $5 \times 10^2 - 5 \times 10^3$ electroporants/μg DNA were obtained for an unidentified methylotroph. Previously, we showed electroporation to be feasible in *M. methylotrophus* AS1, *M. extorquens* AM1, and *M. organophilum* XX although specific electroporation conditions and efficiencies were not indicated *(1)*. To investigate further electroporation in methylotrophs, a comparison of electroporation vs. conjugation for transferring plasmid pHX200V-47-m1 *(17)* (originally constructed as a promoter-probe vector with reporter *xylE*) was made using four methylotrophic strains (*M. methylotrophus* AS1, *M. extorquens* AM1, *M. organophilum* XX, and *Pseudomonas* sp. M27). The impact of polyethylene glycol (PEG) on electroporation was also investigated.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are summarized in Table 1. For cultivating the methylotrophs (*M. methylotrophus* AS1, *M. extorquens* AM1, *M. organophilum* XX, and *Pseudomonas* sp. M27), MacLennan minimal medium (MM) *(18)* supplemented with 0.5% (v/v) methanol (Fisher, Tustin, CA) was used. The cultivation temperature for the methylotrophs was 30°C, with the exception of *M. methylotrophus* AS1, which was grown at 37°C. *E. coli* HB101 and JM109 were cultivated at 37°C in Luria broth (LB) *(19)*. Antibiotics were added to the media at the concentrations shown in Table 1 for strains containing plasmids.

Conjugation and Electroporation of pHX200V-47-m1

The broad-host-range plasmid pHX200V-47-m1 was conjugated into the methylotrophs (recipient) using a tri-parental filter mating method with *E. coli* HB101/pRK2013 (helper) and *E. coli* JM109/pHX200V-47-m1 (donor) *(20)*. This helper strain was grown in the presence of 50 μg/mL kanamycin (Fisher) in LB. The ratio of recipient:donor:helper was 2:1:1 and

Table 1
Bacterial Strains and Plasmids Used in this Study

Strains/plasmids	Characteristics	Antibiotics[a] (μg/mL)	Source/ Reference
Methylotrophs			
M. methylotrophus AS1	Type I	Tet [2]	Hanson *(31)*
M. extorquens AM1	Type II	Tet [4]	Hanson *(31)*
M. organophilum XX	Type II	Tet [15]	Hanson *(31)*
Pseudomonas sp. M27	Type II	Tet [10]	Hanson *(32)*
E. coli			
JM109		Tet [20]	Stratagene
HB101		Kan [50]	H. C. Lim
Plasmids			
pRK2013	tra^+ ColE1 replicon (helper plasmid), Kan^R	—	Lipscomb *(33)*
pHX200V-47-m1	Deletion derivative of pHX200V-47, Tet^R	—	Hanson *(17)*

[a]Concentrations of antibiotics (shown in brackets) added to growth media for plasmid-containing strains (tetracycline for pHX200V-47-m1 and kanamycin for pRK2013).

has been described previously *(1)*. After the cells were allowed to conjugate on a filter placed cell-side up on a LB-agar plate for 24 h, the cell suspension was resuspended in 1 mL of sterile 100 m*M* potassium phosphate buffer (pH = 7.0). This cell suspension was diluted 10^{-5} in the same buffer and 100 μL was plated onto the agar plates containing the selection medium. Conjugant selection was performed on MM supplemented with 1.5% (w/v) Bacto-Agar (Difco Laboratories, Detroit, MI) and tetracycline (United States Biochemical Corp., Cleveland, OH) at the concentrations shown in Table 1.

For electroporation, 20 mL of exponentially-growing cells (A_{600} = 0.3 – 0.5) were washed three times in 300 m*M* sucrose at 4°C and resuspended in 200 μL of 300 m*M* sucrose. This is a slight modification of the method developed by Smith and Iglewski *(21)*. For electroporation with PEG (United States Biochemical, MW 8000), after the final wash step, 15 μL of sterile 30% PEG in distilled deionized H_2O was added to the cell suspension. This also had the benefit of eliminating pellet formation in strain *Pseudomonas* sp. M27. Previously, electroporation was not satisfactory in

this strain owing to the formation of pellets, which retained ionic species and caused arcing during electroporation *(1)*.

Cells were electroporated directly after preparation and were not frozen. Although high transformation efficiencies have been reported for frozen-cell suspensions in *Pseudomonas aeruginosa (22)*, it has also been reported that repeatedly freezing and thawing of the cell suspension may result in excessive lysis of the bacteria *(23)*. Between 250–750 ng of pHX200V-47-m1 (isolated from *E. coli* JM109/pHX200V-47-m1 using a plasmid midi-prep from Qiagen, Chatsworth, CA) were added to 40 μL of competent cells, and the cells were electroporated in a 4°C 0.1 cm electroporation cuvet from Bio-Rad Laboratories (Hercules, CA) with a Gene Pulser and a Pulse Controller unit (Bio-Rad Laboratories) at 25 μF. Resistances varied between 200–600 Ω, and field strengths varied between 11–15 kV/cm. After electroporation, 1 mL of MM supplemented with 0.5% methanol (v/v) (Fisher) was immediately added to the cuvet, and the contents were transferred to a microcentrifuge tube and placed in a 37°C hot block for *M. methylotrophus* AS1 and 30°C hot block for the other methylotrophs. After a membrane recovery time of 12 h, 100 μL were plated on agar (1.5%, w/v) media (same as incubation media) containing tetracycline (Table 1). After the colonies grew to 2–3 mm diameter (3–7 d), colonies were inoculated into liquid media with tetracycline, and plasmid miniprep using the modified acetone-alkaline-lysis procedure of Kim and Lidstrom *(24)*, followed by horizontal gel electrophoresis *(25)* of the *Eco*R I-cleaved pHX200V-47-m1, was used to verify successful electroporation or conjugation.

RESULTS AND DISCUSSION

Recombinant Methylotrophic Strains Created Through Conjugation

Plasmid pHX200V-47-m1 was conjugated successfully into the methylotrophs using triparental mating, and the same conditions were used for each strain. Although it is difficult to quantify the efficiency in which conjugal transfer of plasmid DNA takes place, it is possible to qualitatively compare the efficiency at which different strains uptake this DNA by counting the number of colonies which arise after conjugation. After plating the cell suspension for each strain (described earlier), it was found that the conjugation efficiencies in ascending order were *M. methylotrophus* AS1, *M. organophilum* XX, *M. extorquens* AM1, and *Pseudomonas* sp. M27. Each conjugation selection plate contained 58, 77, 116, and 117 colonies, respectively.

Table 2
Electroporation Results

Strain	Field Strength (kV/cm)	DNA Conc. (ng/μL comp. cells)	Trans-formation Efficiency (electroporants/ μg DNA)	(τ) Time Constant (ms)	Resis. (Ω)	PEG Addition (v/v)
M. methylotrophus						
AS1	11	6.25	0	3.8	200	0
	11	6.25	0	6.0	400	0
	11	6.25	0	8.8	600	0
	12.5	12.5	0	3.9	200	0
	12.5	12.5	0	7.2	400	0
	15	18.75	2.5×10^2	4.2	200	0
	15	18.75	1.3×10^3	4.7	200	0.02%
M. extorquens						
AM1	12.5	12.5	0	4.7	200	0
	12.5	12.5	0	9.2	400	0
	15	18.75	5.0×10^2	4.7	200	0
	15	18.75	1.5×10^2	13.1	600	0
	15	18.75	2.5×10^3	4.2	200	0.02%
M. organophilum						
XX	11	6.25	0	4.2	200	0
	11	6.25	0	6.4	400	0
	11	6.25	0	8.1	600	0
	12.5	12.5	0	4.4	200	0
	12.5	12.5	0	7.9	400	0
	12.5	12.5	0	9.9	600	0
	15	18.75	8.0×10^2	4.6	200	0
	15	18.75	3.3×10^2	12.5	600	0
	15	18.75	5.3×10^3	4.7	200	0.02%
Pseudomonas						
sp. M27	11	6.25	0	arc	200	0
	11	6.25	0	arc	400	0
	11	6.25	0	arc	600	0
	15	18.75	0	arc	200	0
	15	18.75	0	arc	400	0
	15	18.75	0	arc	600	0
	15	18.75	1.2×10^4	4.6	200	0.02%
	15	18.75	8.0×10^3	11.6	600	0.02%

Recombinant Methylotrophic Strains Created Through Electroporation

The electroporation conditions were varied to create the recombinant methylotrophic strains: field strength was varied between 11–15 kV/cm, the theoretical time constant (τ) was varied between 5–15 ms by setting the resistance between 200 Ω and 600 Ω, and the DNA concentrations ranged from 250–750 ng. As shown in Table 2, only high field strengths and con-

centrations of pDNA (15 kV/cm, 19 ng/μL) caused the formation of electroporants. Electroporants were created at both high and low time constants, although a theoretical $\tau = 5$ ms was optimal. It has been previously found that in *P. aeruginosa* the transformation efficiency increased with field strength *(26)*, which agrees well with the results presented here. Compared to conjugation, electroporation had the advantage of requiring fewer steps (no conjugation step was needed), fewer strains (no donor or helper strains were needed), and was quicker (about 12 h).

The addition of PEG resulted in a five- to six-fold increase in the electroporation efficiency (Table 2). PEG has long been used to induce DNA uptake by protoplasts in *Bacillus subtilis (27)* and *Streptococcus faecalis (28)*, and PEG has increased the electroporation efficiency for gram-positive *Enterococcus faecalis* protoplasts (efficiency increased from less than 10^1 to 2.6×10^3 *[29]*). It has also been previously reported that PEG increases the transformation efficiency in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* during electroporation, and that PEG not only extends the length of time the electroporated cells remain permeable but further increases their permeability *(30)*. During incubation with PEG, the pores created during electroporation also apparently grow in size for these two strains; this combined effect of PEG on the size of the pores and their duration may enhance the uptake of DNA *(30)*. These effects, combined with the surfactant action that kept the cells from forming pellets, most likely facilitated the formation of electroporants in *Pseudomonas* sp. M27 in this study and would account for the much higher electroporation efficiency seen in the other methylotrophs.

By increasing the electroporation efficiency in these strains, the utility of this technique extends beyond simple DNA transfer. With high electroporation efficiency, it is possible to evaluate easily libraries of mutations contained on plasmids. This, combined with an efficient selection/screening technique, provides an efficient method for positive mutation selection.

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