Genetic Transformation in *Methylobacterium organophilum*

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SUMMARY

Several mutants have been isolated from the facultative methylotroph, *Methylobacterium organophilum*, using either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ultraviolet light as mutagens. One of these isolates, a glutamate auxotroph lacking isocitrate dehydrogenase, has been transformed to prototrophy, using wild-type DNA, at a frequency of 0.5 %. Competence and DNA uptake occur only in cultures which are near the end of exponential growth, and maximal transformation requires a DNA concentration of 100 $\mu\text{g ml}^{-1}$.

INTRODUCTION

Methylobacterium organophilum is a facultative methylotroph. It can utilize C_1 compounds as sole carbon and energy sources but unlike all previously isolated methane oxidizers it can also grow on more complex organic substrates (Patt *et al.*, 1974). The isolation and characterization of this organism has greatly increased the potential for physiological studies of sterol synthesis (T. E. Patt, unpublished results), membrane biogenesis and C_1 metabolism. However, these studies can only be adequately supported with the benefit of genetic analysis. This depends on the availability of mutants, but mutants of methane oxidizers have been difficult to obtain for reasons that are not clear (Williams & Bainbridge, 1971).

METHODS

Organism. *Methylobacterium organophilum* (ATCC27886, Patt *et al.*, 1974) was used in all experiments.

Media and growth conditions. The modified nitrate mineral salts medium (NMS) previously described (Patt, Cole & Hanson, 1976) was used for all studies. All supplements except methanol were dissolved in distilled water, sterilized separately and added directly to the growth medium at the following concentrations (w/v): succinate, 0.1 %; Casamino acids, 0.05 %; L-glutamate, 0.05 % (unless stated otherwise). Methanol (96 %, v/v) was filter-sterilized and added separately to the growth medium at a concentration of 0.5 % (v/v).

All cultures were incubated at 30 °C with methanol as the carbon source unless stated otherwise. Liquid cultures were incubated on rotary shakers. The large batches of cells used for DNA isolation were grown and harvested as described previously (O'Connor & Hanson, 1975).

Survival curves

Ultraviolet (u.v.) light. An exponential-phase culture (40 ml) was harvested and resuspended in 2 ml sterile NMS medium. The suspension was placed in a sterile Petri dish in the dark and exposed to u.v. light (Westinghouse Sterilamp, no. 782L-30) at a distance of 40 cm. At intervals, samples were withdrawn and plated on NMS/methanol agar.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG). An exponential-phase culture (40 ml) was centrifuged, washed and resuspended in 6 ml NMS/methanol medium. The suspension was divided into 1 ml samples and different amounts of NTG were added to each. The samples were incubated in test-tubes on a shaker for 2 h. From each sample, 0.5 ml was filtered on a Millipore filter (HAWG, 0.45 μ m pore size) and organisms were washed with 10 ml distilled water, resuspended in 0.5 ml sterile NMS medium and plated on NMS/methanol agar.

Penicillin. A late exponential-phase culture was harvested, washed twice with NMS medium, and resuspended in NMS/methanol plus 7.5 % (w/v) polyethylene glycol plus 0.25 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The suspension was divided into five parts and one sample was plated on NMS/methanol agar. Different amounts of penicillin were added to the other four samples and the flasks were incubated for 20 h. Penicillinase (12.5 μ g ml⁻¹; Calbiochem) was added and incubation was continued for 1 h. The bacteria were then washed and samples were spread on the surface of NMS/methanol agar plates.

DNA isolation. Frozen cells were thawed, washed twice in 0.05 M-Tris/0.02 M-EDTA (TE) buffer, pH 8.0, and resuspended in 90 ml TE buffer per 5.0 g wet wt cells. The solution was made up to 1 % (w/v) in sodium lauryl sulphate and heated to 60 °C for 30 min. Ten ml *Streptomyces griseus* type VI protease (5 mg ml⁻¹; Sigma), previously heat-treated at 80 °C for 10 min, was added and incubation was continued at 60 °C for 2 h or until lysis was complete. Water-saturated phenol (0.5 vol.) was added and the solution was shaken for 15 min. The solution was then centrifuged and the aqueous layer removed. RNAase A and RNAase T₁ (Sigma), previously heated in boiling water for 5 min, were added to a final concentration of 100 μ g and 30 units ml⁻¹, respectively, and the solution was incubated at 37 °C for 1 h. The DNA was precipitated with 2 vols cold 95 % ethanol, and resuspended in sterile 0.1 $\times$ standard saline citrate buffer (SSC: 0.15 M-sodium chloride/0.015 M-sodium citrate, pH 7.3). DNA preparations were kept frozen in 0.5 ml quantities at -20 °C. DNA concentrations were estimated using the diphenylamine assay described by Burton (1956).

Sheared DNA was prepared by vortexing the sample for 30 s at high speed, then forcing it through a 26½ gauge needle five times. This greatly diminished the viscosity of the DNA solution.

Determination of the molecular weight of DNA. The average size of sheared and un-sheared DNA preparations was estimated using an agarose gel electrophoresis technique similar to that of Helling, Goodman & Boyer (1974). Lambda DNA, treated with EcoRI nuclease, was used as a standard. Agarose was obtained from MCK Biomedical (Rockland, Maine, U.S.A.).

Mutant isolation.

Mutagenesis. (i) U.v. light. Bacteria were prepared and exposed to u.v. light for 3 min as described for the survival curve. The mutagenized bacteria were transferred to a stirred flask of supplemented medium and incubated for 8 to 12 h at room temperature in the dark.

(ii) NTG. A culture (40 ml) was treated as described for the NTG survival curve. A sample (1 ml) was incubated on a shaker for 25 min, 1 ml filter-sterilized NTG (4 mg ml⁻¹) was added and incubation was continued for 2 h. The bacteria were collected on a Millipore filter and washed thoroughly with NMS medium. The filter was placed in 40 ml supplemented medium and incubated on a shaker overnight.

Penicillin selection. Mutagenized, overnight cultures were washed twice with sterile NMS media and resuspended in nitrogen-free NMS medium at a density of 10⁶ viable units ml⁻¹.

After 10 h incubation on a shaker, the culture was transferred to minimal NMS medium containing 7.5 % polyethylene glycol, 0.25 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 500 units penicillin ml^{-1} (Sigma) and was incubated for a further 20 h. Penicillinase was then added to a final concentration of $12.5 \mu\text{g ml}^{-1}$, and incubation was continued for another hour.

Mutant selection. Three types of mutants were selected: amino-acid auxotrophs, drug-resistant mutants, and mutants unable to grow on methanol but capable of growth on succinate (methanol⁻ mutants). The mutagenized bacteria from the penicillin selection treatment were washed and plated on supplemented media. For the drug-resistant mutants, colonies which grew on NMS/methanol medium plus streptomycin ($50 \mu\text{g ml}^{-1}$), erythrocine ($500 \mu\text{g ml}^{-1}$) or rifampicin ($50 \mu\text{g ml}^{-1}$), were picked and streaked for isolation. For the auxotrophs, colonies on the supplemented plates (NMS/methanol medium plus 0.05 % Casamino acids) were replica-plated on to unsupplemented media (NMS/methanol) and those which grew on the supplemented media but not on the unsupplemented were purified by streaking on supplemented media. Methanol⁻ mutants were selected as colonies which grew on NMS plus succinate agar but would not grow when replicated on to NMS/methanol agar.

Preparation of extracts. Late exponential-phase cultures were harvested, washed and resuspended in TMME buffer (Hanson & Cox, 1967) using 2 ml buffer per g cells. The bacteria were sonicated at maximum intensity for 3 min (Bronwill Biosonik Sonifier, Bronwill Scientific, Rochester, New York, U.S.A.), and centrifuged at 20000 g for 20 min. The soluble fraction was assayed for isocitrate dehydrogenase activity. Protein was estimated by the method of Lowry *et al.* (1951).

Isocitrate dehydrogenase assay. Isocitrate dehydrogenase (EC. 1.1.1.42) was assayed by the method of Hanson & Cox (1967). NADP^+ (3 mM) was incubated in a cuvette with cell-free extract for 1 min to ensure there was no NADP^+ reduction in the absence of substrate. Isocitrate (1.5 mM final concentration) was added and the activity was followed by measuring the change in E_{340} in a Gilford 2000 Spectrophotometer (Gilford Laboratories Instruments, Oberlin, Ohio, U.S.A.).

Growth response of mutant 3A to L-glutamate. Tubes containing 3 ml NMS/methanol medium and different amounts of L-glutamate, were inoculated with isolate 3A to give an initial absorbance of about 0.15 at 600 nm and were incubated with shaking. The absorbance of samples taken from the tubes was determined at 24 h intervals.

Transformation

DNA present throughout growth. For routine experiments, DNA from wild-type bacteria ($100 \mu\text{g ml}^{-1}$) was added to inoculated cultures which were allowed to grow to stationary phase. The bacteria were washed and plated on selective (NMS/methanol) and non-selective (NMS/methanol plus Casamino acids) media. In some cases, the bacteria were regrown for two generations in the presence of glutamate before plating.

For all experiments, control cultures were included containing sterile $0.1 \times \text{SSC}$ in place of DNA, or DNA which had been treated with $40 \mu\text{g DNAase ml}^{-1}$ for 30 min before addition to the culture.

Competence and DNA uptake determinations. Triplicate 1 ml samples were taken from a culture at various times during growth, measured using a Klett–Summerson colorimeter (Klett Manufacturing Co., New York, U.S.A.). Two of the samples were used to determine competence: one was incubated for 1 h with $100 \mu\text{g}$ wild-type DNA, while the other was incubated with an equivalent volume of $0.1 \times \text{SSC}$ as a control. DNAase (final concentration

Fig. 1

Fig. 2

Fig. 1. Viable units remaining after treatment of cultures with mutagens or penicillin: ▲, u.v. light; ●, penicillin (20 h incubation); ■, NTG (2 h incubation). Initial concentrations (viable units ml⁻¹) were 4×10^8 (u.v.), 1.2×10^8 (penicillin) and 3×10^8 (NTG).

Fig. 2. Growth response of wild type and isolate 3A to glutamate. The growth medium was NMS/methanol. ▲, Wild type, all concentrations of glutamate (0 to 500 µg ml⁻¹). Isolate 3A with glutamate at (µg ml⁻¹): □, 500; ■, 100; ○, 50; +, 10. ●, Isolate 3A, no additions.

20 µg ml⁻¹) was added to both samples and incubation was continued for 30 min. The samples were then plated on selective and non-selective media. The third sample was used to determine DNA uptake. Wild-type tritiated DNA (10 µg; 20000 c.p.m. µg⁻¹; prepared by T. E. Patt, as described earlier, Patt *et al.*, 1974) was added to the sample and incubated for 1 h before DNAase (final concentration 20 µg ml⁻¹) was added and incubation was continued for 30 min. The samples were filtered on Millipore filters and washed thoroughly with distilled water. The filters were dried, 10 ml Bray's scintillation fluid was added (Bray, 1960) and the radioactivity was assayed in a Packard model 3375 scintillation spectrophotometer.

RESULTS

Survival curves. The sensitivity of *Methylobacterium organophilum* to NTG, u.v. radiation and penicillin treatment is shown in Fig. 1. The NTG concentration chosen for mutagenesis was 2 mg ml⁻¹ which gave 99.7 % kill in 2 h. The time of exposure chosen for u.v. mutagenesis (180 s) gave 92 % kill.

Mutant isolation. Several mutants were isolated using either NTG or u.v. mutagenesis, followed by penicillin selection. The frequency of mutant isolation was about 5 per 10⁵ colonies screened, except drug-resistant mutants, which appeared at a frequency of about 1 per 10⁷. The isolated mutants included auxotrophs, drug-resistant mutants and mutants unable to grow on methane or methanol, but capable of growth on glucose or succinate. Twenty-seven mutants were isolated using NTG, 24 using u.v. light. Many of the isolated mutants were considered too leaky for use in transformation studies. One of the auxo-

Table 1. *Isocitrate dehydrogenase assay of isolate 3A and wild type*

Specific activities are expressed as nmol NADP⁺ reduced min⁻¹ (mg protein)⁻¹.

Crude extract (mg protein) added to reaction mixture		Specific activity
3A	Wild type	
0.35	0	0
0	0.29	28
0.35	0.29	34

Table 2. *Transformation frequencies*

Bacteria (isolate 3A) were incubated with DNA throughout the growth cycle. Transformation frequencies are expressed as the ratio of the no. of glutamate⁺ transformants to the total no. of bacteria.

Transformation conditions	Transformation frequency
3A + wild-type DNA (100 µg ml ⁻¹)	5.3×10^{-3}
3A + wild-type DNA (100 µg ml ⁻¹), bacteria regrown for two generations with glutamate	6.1×10^{-3}
3A + wild-type DNA (100 µg ml ⁻¹) pretreated with DNAase	4.1×10^{-6}
3A + 0.1 × SSC (no DNA)	1.2×10^{-6}

trophic isolates obtained by NTG mutagenesis was designated 3A and was chosen for further study.

Growth requirements of isolate 3A. Isolate 3A required glutamate for growth (Fig. 2). As the concentration of glutamate was increased, the growth rate and final cell densities increased to a maximum at 500 µg glutamate ml⁻¹. Higher concentrations of glutamate did not result in further growth stimulation. At its maximum growth, isolate 3A did not grow as well as the wild type. Although it grew slowly in the absence of glutamate, it formed only tiny colonies on NMS/methanol agar. Mutant colonies were readily distinguished from the larger colonies of wild-type bacteria.

Isolate 3A was always filled with poly-β-hydroxybutyrate granules which permitted simple identification of the mutant and wild-type bacteria under the microscope. Because the isolate grew more slowly than the wild type, revertants, when present, outgrew the mutants after several transfers. Cultures of mutants were therefore always restreaked to obtain isolated colonies before each experiment.

Enzymic deficiency in isolate 3A. Isolate 3A lacks isocitrate dehydrogenase activity, which explains its requirement for glutamate. When an extract of the isolate was incubated with NADP⁺ and isocitrate, there was no activity (Table 1). When wild-type extract was added to the same reaction mixture, the activity observed was similar to that of the wild-type extract alone. Therefore, the lack of activity in the mutant extract was not due to the presence of an inhibitor.

Transformation frequencies. When cultures were incubated with DNA throughout their growth cycle, transformation frequencies of about 0.5 % were obtained (Table 2). The frequency of transformation was increased by only 13 % when transformed cultures were regrown in the presence of glutamate for two more generations; thus, phenotypic lag did not significantly influence the transformation frequency.

Cultures containing DNA which had been pretreated for 30 min with 40 µg DNAase ml⁻¹ gave frequencies of about 4×10^{-6} . The observed reversion frequency was similar.

Fig. 3

Fig. 4

Fig. 3. The dependence of transformation frequency on DNA concentration. Cultures of isolate 3A were grown to stationary phase in the presence of sheared (■) or unsheared (●) DNA from wild-type *M. organophilum* and portions of the culture were spread on plates of NMS/methanol or NMS/methanol plus Casamino acids.

Fig. 4. The development of competence and the uptake of DNA in isolate 3A. Samples were taken at various times during growth and incubated for 1 h with either 100 μg wild-type DNA ml⁻¹ (for determination of transformation frequencies) or 10 μg wild-type [³H]DNA ml⁻¹ (for determination of DNA uptake). ●, Growth; ○, frequency of transformants; ■, DNA uptake.

DNA requirement for transformation. A DNA concentration of 100 μg ml⁻¹ was necessary for maximum transformation frequencies (Fig. 3). The use of sheared DNA resulted in slightly lower transformation frequencies. The sizes of the sheared and unsheared DNA preparations were approximately 7×10^6 daltons (range: 6.5×10^6 to 7.5×10^6 daltons) and 23×10^6 daltons (range: 19×10^6 to 28×10^6 daltons) respectively.

Deoxyribonuclease assay. DNAase activity in the culture supernatant was determined by incubating samples with tritiated DNA, precipitating with cold trichloroacetic acid, and counting the radioactivity in the precipitates. No DNAase activity was detected.

Competence and DNA uptake. The ability to be transformed occurred only in a narrow portion of the growth cycle, near the end of exponential growth (Fig. 4). DNA uptake occurred at the same point in the growth cycle as transformation. Maximum DNA uptake in the wild-type cells was only 14 % of that in isolate 3A and transformation of a streptomycin resistance marker into the wild type occurred at a frequency of 4×10^{-5} .

Transformation of a double mutant. A streptomycin-resistant mutant of isolate 3A was isolated which grew in the presence of 50 μg streptomycin ml⁻¹ and required glutamate for growth. This double mutant was transformed to a streptomycin-resistant prototroph at a frequency of 0.1 %.

DISCUSSION

Normal mutagenesis procedures resulted in the isolation of several mutants in *M. organophilum*. The frequency of mutant isolation was low compared to *Escherichia coli* (Adelberg, Mandel & Chein Ching Chen, 1965), but higher than that reported by Williams

& Bainbridge (1971) for the obligate methane oxidizer, *Methylococcus capsulatus*. Initially high doses of NTG were used to ensure the isolation of mutants but the u.v. light procedure also resulted in the isolation of mutants. Ultraviolet light is considered the more desirable of the two mutagens because of its relative safety and the tendency of NTG to produce multiple mutations in bacteria (Guerola, Ingraham & Cerda'-Almedo, 1971).

Isolate 3A takes up more DNA than the wild type and is transformed at higher frequencies than the wild type or other auxotrophs (unpublished results). The transformation frequencies which were obtained compare favourably with those in *Bacillus* (Anagnostopoulos & Spizizen, 1961), *Pneumococcus* (Hotchkiss, 1954), *Haemophilus* (Spencer & Herriott, 1965) and *Methylococcus* (Williams & Bainbridge, 1971). Competence occurs at approximately the same point in the growth cycle for all of these transformation systems. However, the DNA concentration required for maximal transformation is 10 to 100 times higher in *M. organophilum* than in *Bacillus* (Anagnostopoulos & Spizizen, 1961), *Pneumococcus* (Cato & Guild, 1968), or *Haemophilus* (Stuy & Stern, 1964), but is similar to that described for maximal transformation of *Methylococcus* (Williams & Bainbridge, 1971).

Sheared DNA (16×10^6 daltons) gives maximal transformation in *Escherichia coli*, with larger and smaller DNA pieces giving lower transformation frequencies (Cosloy & Oishi, 1973). However, in *Bacillus* (Morrison & Guild, 1972), *Pneumococcus* (Cato & Guild, 1968) and *Haemophilus* (Nicolaiëff & Chevallier, 1970), transformation increases with DNA size from nearly zero at DNA sizes of 0.2×10^6 to 0.4×10^6 daltons to a maximum at 20×10^6 to 30×10^6 daltons, after which it remains constant. In *Bacillus* and *Pneumococcus* the decrease in transformation frequency caused by using DNA of 7×10^6 daltons (the same size as the sheared DNA used in this study) was similar to that observed in *M. organophilum* transformation (Morrison & Guild, 1972; Cato & Guild, 1968).

The absence of DNAase in the culture supernatant during growth allowed incubation of the DNA with the bacteria throughout the growth cycle so that every competent cell was exposed to DNA. When the cells were exposed to DNA for only short periods, maximum transformation frequencies were lower. This was probably due to a lack of synchrony in the development of competence and possibly to phenotypic lag.

The selective transformation of a single marker out of a doubly-marked strain, together with the results for controls without DNA or with DNAase, shows that the counted transformants are not contaminants and suggests that the transformation observed is not due to an enhancement of reversion.

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REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEIN CHING CHEN, G. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788-795.
- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741-746.
- BRAY, G. A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analytical Biochemistry* **1**, 279-285.
- BURTON, K. (1956). A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical Journal* **62**, 315-323.

- CATO, A. & GUILD, W. R. (1968). Transformation and DNA size. I. Activity of fragments of defined size and a fit to a random double crossover model. *Journal of Molecular Biology* **37**, 157-178.
- COSLOY, S. D. & OISHI, M. (1973). The nature of the transformation process in *Escherichia coli* K12. *Molecular and General Genetics* **124**, 1-10.
- GUEROLA, N., INGRAHAM, J. L. & CERDA'-ALMEDO, E. (1971). Induction of closely linked multiple mutations by nitrosoguanidine. *Nature, London* **230**, 122-125.
- HANSON, R. S. & COX, D. P. (1967). Effect of different nutritional conditions on the synthesis of tricarboxylic acid cycle enzymes. *Journal of Bacteriology* **93**, 1777-1787.
- HELLING, R. B., GOODMAN, H. M. & BOYER, H. W. (1974). Analysis of endonuclease R.EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. *Journal of Virology* **14**, 1235-1244.
- HOTCHKISS, R. D. (1954). Cyclical behaviour in pneumococcal growth and transformability occasioned by environmental changes. *Proceedings of the National Academy of Sciences of the United States of America* **40**, 49-55.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MORRISON, D. A. & GUILD, W. R. (1972). Activity of deoxyribonucleic acid fragments of defined size in *Bacillus subtilis* transformation. *Journal of Bacteriology* **112**, 220-223.
- NICOLAIEFF, A. & CHEVALLIER, M. R. (1970). An electron microscopic study of the relation between length and transforming activity of *Haemophilus influenzae*. *Molecular and General Genetics* **109**, 269-277.
- O'CONNOR, M. L. & HANSON, R. S. (1975). Serine transhydroxymethylase isoenzymes from a facultative methylotroph. *Journal of Bacteriology* **124**, 985-996.
- PATT, T. E., COLE, G. C., BLAND, J. & HANSON, R. S. (1974). Isolation and characterization of bacteria that grow on methane and organic compounds as sole sources of carbon and energy. *Journal of Bacteriology* **120**, 955-964.
- PATT, T. E., COLE, G. C. & HANSON, R. S. (1976). *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *International Journal of Systematic Bacteriology* **26**, 226-229.
- SPENCER, H. T. & HERRIOTT, R. M. (1965). Development of competence of *Haemophilus influenzae*. *Journal of Bacteriology* **90**, 911-920.
- STUY, J. H. & STERN, D. (1964). The kinetics of DNA uptake by *Haemophilus influenzae*. *Journal of General Microbiology* **35**, 391-400.
- WILLIAMS, E. & BAINBRIDGE, B. W. (1971). Genetic transformation in *Methylococcus capsulatus*. *Journal of Applied Bacteriology* **34**, 683-687.