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Creating auxotrophic mutants in *Methylophilus methylotrophus* AS1 by combining electroporation and chemical mutagenesis

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Abstract Stable auxotrophic mutants of the methylotroph *Methylophilus methylotrophus* AS1 were obtained by a novel mutagenesis technique in which electroporation is used to transport the chemical mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) across the cell membrane. By combining chemical mutagenesis with electroporation and screening single colonies for auxotrophy in 36 different amino acids and growth factors, 3 auxotrophs per 156 colonies screened were obtained, whereas no auxotrophs were found with chemical mutagenesis alone. MNNG mutagen toxicity was also increased in the methylotroph with this novel mutagenesis technique (death rate 96% compared to 79%). This technique did not increase the mutation rate for strain *Escherichia coli* BK6 which responds well to simple exposure to the mutagen.

Introduction

Methylotrophic bacteria are those capable of growing on one-carbon compounds and have been extensively studied because of their potential commercial value for producing single-cell protein, amino acids, pyrroloquinoline quinone, and poly-β-hydroxybutyrate (Ueda et al. 1991). There have been several genetic studies on methylotrophs, but progress in this area has been slow since it has been difficult to create mutants in methylotrophs (de Vries et al. 1990), and auxotrophic mutants are especially difficult to obtain (Higgins et al. 1980; Moore et al. 1983; O'Connor and Hanson 1978). Although mutants of *Methylophilus methylotrophus* deficient in glutamate synthase have been isolated using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Windass et al. 1980) and non-stable auxotrophs have been obtained (Holloway 1981), no mutation technique has been described that consistently produces stable auxotrophy in this strain. The types of mutagenesis techniques examined in the past include UV irradiation and chemical alkylating agents such as MNNG, methyl methanesulphonate, and ethyl methanesulphonate; most of the mutants obtained in facultative methylotrophs have been induced using MNNG (de Vries et al. 1990). Although ethyl methanesulphonate has had success in mutating *Methylobacterium extorquens* AM1 (Morris et al. 1994), and mutants in methanol utilization have been isolated using UV irradiation in strains *Methylobacterium organophilum* XX (de Vries 1986), mutagenesis of methylotrophs remains infrequent. The ineffectiveness of UV irradiation in methylotrophs has been hypothesized to involve the lack of an SOS repair system (Higgins et al. 1980), and chemical mutagens have not been effective possibly because of their inability to permeate the membrane (de Vries 1986; Holloway et al. 1987).

Electroporation has become a useful technique in the transformation of plasmid DNA into a broad range of prokaryotic cells (Diver et al. 1990; Farinha and Kropinski 1990; Iwasaki et al. 1994; Jahng and Wood 1994; Siguret et al. 1994; Smith and Iglewski 1989; Steele et al. 1994; Ueda et al. 1991). This technique involves subjecting competent cells to a brief high electrical impulse which reversibly permeabilizes cells and allows plasmid DNA to diffuse into the cell (Smith and Iglewski 1989). The membrane is repaired during subsequent growth in complex medium.

On the basis of this successful plasmid DNA mobilization, it seemed reasonable that this method may be a general diffusion system for other molecules that might otherwise have problems permeating the cell membrane because of charge or size. In this work, a novel technique for introducing the chemical mutagen MNNG into *M. methylotrophus* AS1 through electroporation has

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been developed. This method resulted in an increased mutation frequency in this strain as compared to simple exposure to the mutagen.

Materials and methods

Bacterial strains and growth conditions

Methylophilus methylotrophus AS1 is a type I methylotroph and was a generous gift from Professor R. S. Hanson (Tsuji et al. 1990) at the University of Minnesota. This strain was grown in MacLennan minimal medium (MacLennan et al. 1971) containing 0.5% (v/v) methanol (Fisher Scientific, Tustin, Calif.), and colony growth of these cells was characterized by a distinct reddish hue. *Escherichia coli* BK6 [Δ(*lacIPOZ*)*C29*, *lacY*$^{+}$, *hsdR*, *galU*, *galK*, *strA*^{r}, *leuB6*, *trpC9830*, Δ(*srl-recA*)306::Tn*10*] was obtained from laboratory stocks (Wood et al. 1990). The growth medium for this strain was Luria-Bertani medium (LB) (Sambrook et al. 1989) and was cultured at 37 °C. Exposure to MNNG (Aldrich Chemical Co., Milwaukee, Wis.) and cell membrane recovery were performed in M9 medium (Rodriguez and Tait 1983) supplemented with 0.4% (w/v) casamino acids (Difco Laboratories, Detroit, Mich.) and 0.5% (v/v) methanol (Fisher Scientific) (M9CM medium). All screening was performed in M9 minimal medium supplemented with 0.4% (w/v) glucose (Fisher Scientific) and 36 different amino acids and growth factors as described by Holliday (1956). Amino acids and growth factors were purchased from United States Biochemical Corp. (Cleveland, Ohio) with the exception of L-asparagine and L-proline, which were purchased from Fisher Scientific, and L-glutamic acid, DL-ornithine, L-phenylalanine, L-tyrosine, and L-valine, which were purchased from the Sigma Chemical Co. (St. Louis, Mo.). All solid media contained the liquid medium supplemented with 1.5% (w/v) Bacto-Agar (Difco Laboratories).

Mutagenesis by electroporating *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

A 20-ml sample of exponentially growing cells (A_{600} = 0.3–0.5) was washed three times in 300 mM sucrose at 4 °C and resuspended in 200 µl 300 mM sucrose (4 °C). This is a slight modification of the method developed by Smith and Iglewski (1989) and has been used previously in this laboratory (Jahng et al. 1996). A 100-µg sample of a 10-mg/ml stock solution of MNNG, dissolved in dimethylsulphoxide (Fisher Scientific) and stored at 4 °C, was added to 80 µl of these sucrose-washed cells, and the cells were electroporated in a 4 °C 0.2-cm electroporation cuvette from Bio-Rad Laboratories (Hercules, Calif.) with a Gene Pulser and a Pulse Controller unit (BioRad Laboratories) at 25 µF, 200 Ω, and 11 kV/cm. After electroporation, 1 ml M9CM for *M. methylotrophus* AS1 and LB for *E. coli* BK6 was immediately added to the cuvette and the contents were transferred to a microcentrifuge tube and placed in a 37 °C hot block. After allowing for cell membrane repair and additional exposure to the mutagen (5 h or 12 h for *M. methylotrophus* AS1 and 30 min for *E. coli* BK6), these cell suspensions were diluted and plated on M9CM/agar plates for *M. methylotrophus* AS1 and LB/agar plates for *E. coli* BK6. After growth at 37 °C, colonies were counted, and the percentage of cells killed through exposure to the mutagen and electroporation were determined by comparing the number of viable cells after electroporation in the presence of MNNG and the number of viable cells after electroporation only to the number of viable cells originally present.

Auxotrophic screening

The Holliday selection procedure (Holliday 1956) screens for auxotrophy in 36 different growth factors (each made aseptically in sterile distilled water and stored at 4 °C) using 12 different M9/agar plates (pools) supplemented with 0.4% (w/v) glucose and growth factors. A slight modification of this procedure was used for screening mutants of *E. coli* BK6 (*leuB6*, *trpC9830*). Since *E. coli* BK6 is already auxotrophic in leucine and tryptophan, all pools were supplemented with leucine and tryptophan. This had the result of screening for 34 growth factors for *E. coli* BK6 mutants as opposed to 36 for *M. methylotrophus* AS1.

To corroborate the auxotrophy identified by growth on the Holliday selection plates, mutant colonies were cultured in M9 minimal medium supplemented with 0.5% methanol and the prospective specific amino acids and growth factors being tested. These potential auxotrophs were also grown in M9 minimal medium and 0.5% methanol (M9M medium) with no amino acid or growth factor additions to determine the extent of mutation for these strains.

Results

Three stable auxotrophic mutants of *M. methylotrophus* AS1 were created using the electroporation/mutagenesis treatment with MNNG, and they remained stable after 2 weeks of growth on M9/agar plates supplemented with 0.4% glucose and the growth factors in question (Table 1). After 5 h of MNNG exposure and membrane repair, 52 colonies were screened for 36 different growth factors using the Holliday selection technique, and one mutant auxotrophic in folic acid was found (Table 1). By increasing the MNNG exposure and membrane repair time to 12 h, two additional auxotrophic mutants (one polyauxotrophic in serine and alanine, and another polyauxotrophic in glutamic acid and inositol) were found after screening 104 colonies (Table 1).

As a control, sucrose-washed cells were also treated with MNNG but not electroporated, the other conditions being identical to those for the electroporation/mutagenesis treatment (MNNG exposure time of 12 h). A total of 156 colonies were screened with the Holliday pools and none displayed auxotrophy for the 36 growth factors. One additional negative control was performed in which sucrose-washed cells were electroporated without the mutagen, and screened for mutations. Of the 104 additional colonies screened, none displayed auxotrophy.

Thousands of competent cells were also plated in serial dilutions onto the M9CM/agar plates to determine the cell survival rate after the different treatments described. MNNG with no electroporation resulted in a 42.3% death rate of *M. methylotrophus* AS1 after 12 h of exposure in M9CM (relative to no MNNG exposure or electroporation), whereas exposure to the MNNG with electroporation resulted in a 96% death rate after 12 h in the same medium. Electroporation alone resulted in a 79% death rate of the methylotrophs compared to untreated sucrose-washed cells. This result suggests that electroporation leads to enhanced diffusion of MNNG into the cell.

Further screening of the auxotrophic mutants of *M. methylotrophus* AS1 in liquid culture confirmed the results obtained with the Holliday selection plates. It

Table 1 Auxotrophic mutants created in this study. The death rate was calculated by comparing the number of viable cells after the treatment described to the number of viable cells receiving no treatment. *MNNG N*-methyl-*N′*-nitro-*N*-nitrosoguanidine

Strain	Treatment	Auxotrophy	Number of colonies screened	Death rate (%)	Mutant name
M. methylotrophus AS1	Electroporation/ MNNG (5 h)	Folic acid	52	95.6	*M. methylotrophus* AS1 DGF1
M. methylotrophus AS1	Electroporation/ MNNG (12 h)	Serine and alanine	104	96.0	*M. methylotrophus* AS1 DGF2
		Glutamic acid and inositol			*M. methylotrophus* AS1 DGF3
M. methylotrophus AS1	MNNG alone (12 h)	None	156	42.3	
M. methylotrophus AS1	Electroporation alone	None	104	78.7	
E. coli BK6	Electroporation/ MNNG (30 min)	Serine and glycine	156	99.8	*E. coli* BK6 DGF1a
		Arginine and ornithine			*E. coli* BK6 DGF2
		Cysteine and sodium thiolate			*E. coli* BK6 DGF3
		Biotin			*E. coli* BK6 DGF4
		Cytosine			*E. coli* BK6 DGF5
E. coli BK6	MNNG alone (30 min)	Serine and glycine	156	99.8	*E. coli* BK6 DGF1b
		Glycine (3 mutants)			*E. coli* BK6 DGF6a–c
		Phenylalanine			*E. coli* BK6 DGF7
		Folic acid			*E. coli* BK6 DGF8
E. coli BK6	Electroporation alone	None	104	76.8	

should be noted that, in liquid culture, the *M. methylotrophus* AS1 mutants were able to grow very slowly (compared to the wild-type strain) in minimal medium that lacked the auxotrophic compound. This may be due to leaky mutations or crossfeeding of necessary metabolites.

There was no significant benefit of adding electroporation to the MNNG mutagenesis treatment for *E. coli* BK6 since MNNG is much more toxic to this strain: 100% of the cells exposed to MNNG for 1 h were killed; hence, the MNNG exposure and membrane recovery time were reduced to 30 min. Five auxotrophic mutants were obtained with MNNG mutagenesis combined with electroporation while six auxotrophic mutants were found with the control of MNNG treatment alone (Table 1). Both simple exposure to MNNG for 30 min and MNNG treatment combined with electroporation resulted in a 99.8% death rate (relative to no MNNG exposure or electroporation); therefore, there was no increase in toxicity. For the auxotrophic mutants of *E. coli* BK6 in liquid culture, the results obtained with the Holliday selection plates were confirmed.

Discussion

Since MNNG has been successfully used to create several mutant *E. coli* (Ito et al. 1994; Reed and Hutchinson 1987; Sato et al. 1991), it is reasonable that electroporation does not increase the mutation rate for this strain since there seems to be no difficulty in MNNG diffusion across the *E. coli* membrane. However, for *M. methylotrophus* AS1, the results presented here suggest that the main difficulty in mutating this strain lies in the chemical mutagen diffusing across the cell membrane, as suggested by de Vries et al. (1990) and Holloway et al. (1987).

Since electroporation is an effective transformation procedure for the introduction of plasmids into methylotrophs (Jahng et al. 1996), and the basis of electroporation is to compromise the integrity of the cell membrane so that plasmid DNA may diffuse into the cell, the results shown here indicate that this same procedure may be used to introduce MNNG into the methylotrophs, and thus increase the mutation rate. It also seems reasonable that this procedure would work with other strains that are difficult to mutagenize as well as with other chemical mutagens (e.g. methyl methanesulphonate and ethyl methanosulphonate) and many other chemicals.

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