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Construction of insertion and deletion *mxa* mutants of *Methylobacterium extorquens* AM1 by electroporation

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Abstract

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph which is widely used for analyzing pathways of C_1 metabolism with biochemical and molecular biological techniques. To facilitate this approach, we have applied a new method to construct insertion or disruption mutants with drug resistance genes by electroporation. By using this method, mutants were obtained in four genes present in the *mxa* methylotrophy gene cluster for which the functions were unknown, *mxaR, mxaS, mxaC* and *mxaD*. These mutants were unable to grow on methanol except the mutant of *mxaD*, which showed reduced growth on methanol. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Methylobacterium extorquens* AM1; Methylotroph; Methanol oxidation; Electroporation

1. Introduction

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph which was originally isolated as an airborne contaminant of liquid methylamine culture [1]. This microorganism can grow on C_1 compounds such as methanol and methylamine using the serine cycle for carbon assimilation, but it is unable to grow on methane. It also grows on ethanol and some organic acids as a sole carbon and energy source. This organism has been well characterized physiologically and biochemically [2], and its molecular genetics is well established. A number of chromosomal regions of *M. extorquens* AM1 have been characterized that are involved in C_1 metabolism, showing that many C_1 genes are clustered [3–5]. The function of many of these genes has been determined by a combination of mutant and gene sequence analysis. However, a subset of these clustered genes have unknown functions, as they show little or no similarity to other proteins or show similarity to proteins with unknown function in protein databases.

Constructing mutants in open reading frames is

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an important step in elucidating the function of unknown genes. The usual approach in *M. extorquens* AM1 is an allelic exchange procedure, which involves inserting a drug resistance gene into the open reading frame of interest, and exchanging this with the chromosomal gene copy via homologous double-crossover recombination events [3]. This approach is cumbersome, because it involves two subsequent subcloning steps to obtain the construction for generating the allelic exchange, and the mating step requires purification of the recipient from the donor, even in the presence of a counter-selective marker. In addition, the majority of the drug-resistant strains are the result of single-crossover recombination events, and in many cases a large number of recombinants must be screened for loss of the vector drug resistance marker to obtain the desired double-crossover recombinants.

Allelic exchange mutagenesis with linear fragments is a more attractive procedure for generating insertional mutants in strains such as *M. extorquens* AM1, as it simplifies both the cloning and mutation processes. However, until now there has been no published procedure for transferring linear DNA fragments into *M. extorquens* AM1. Procedures are available for electroporation of closed circular plasmids [6,7]. Therefore, we have adapted these procedures for the electroporation of linear DNA fragments into *M. extorquens* AM1 to generate chromosomal insertion mutants by allelic exchange.

2. Materials and methods

Strains and plasmids used are listed in Table 1. *M. extorquens* AM1 rif [8] was used as the wild-type strain and for mutation.

2.1. Growth conditions

The growth medium originally described by Attwood and Harder [9] was used for *M. extorquens* AM1. Methanol, methylamine hydrochloride or sodium succinate were used as carbon sources, at 0.5% (v/v), 0.2% (w/v) and 0.46% (w/v), respectively. *Escherichia coli* was grown on LB medium [10]. Antibiotics used were: kanamycin, 25 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; ampicillin, 50 μg ml^{-1} for *E. coli*, 300 μg ml^{-1} for *M. extorquens* AM1.

2.2. Electroporation

Electro-competent cells were prepared as follows. *M. extorquens* AM1 was grown to exponential phase on succinate medium. The culture was chilled for 15 min on ice and centrifuged at 2000 rpm at 4°C for 10 min. Cells were washed with ice-cold sterile distilled water, then washed with ice-cold sterile 10% (v/v) glycerol. Cells were suspended in 10% glycerol, dispensed in 100-μl aliquots and kept at −70°C. Electro-competent cells (50 μl) were mixed with DNA solution (2 μl) and transferred into a cuvette chilled on ice. Electroporation was carried out using a Gene Pulser (Bio Rad) with the following parameters: 2.5 kV, 400 Ω, 25 μF for 2-mm gap cuvette, or 2 kV, 200 Ω, 25 μF for 1-mm gap cuvettes. After the cells had been pulsed, ice-cold sterile Nutrient Broth (Difco) was added to the cuvette immediately, the cell suspension was transferred into a test tube, and it was then shaken at 30°C for 1–24 h. An aliquot was spread on an agar medium with succinate and appropriate antibiotics. Colonies usually appeared after 3 days.

2.3. DNA manipulations

Plasmid DNA from *E. coli* strains was prepared as described by Sambrook et al. [10]. Restriction enzyme digestion, DNA ligation and other DNA modifications were performed according to vendors' recommendations.

2.4. DNA hybridization

Chromosomal DNA was isolated by the method of Marmur [11] with slight modifications. Chromosomal DNA (5 μg) digested with restriction enzyme(s) was separated by agarose gel electrophoresis, blotted onto a nylon membrane and hybridized with an appropriate probe. Labeling of the probe and detection of hybridized DNA were done with DIG labeling and detection kit (Boehringer Mannheim). Probes used were pUC18 and the 1.2-kb *Eco*RI fragment from pUC4K.

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)	Source or reference
E. coli strains		
DH5α	r-m+ *recA1 lacZYA* ϕ80 d*lac*Δ(*lacZ*)*M15*	Bethesda Research Laboratories
S17-1	*recA oriT* integrated RP4-2-Tc::Mu-Km::Tn*7*	[21]
JM110	*dam dcm supE*44 *hsdR*17 *thi leu rpsL lacY* F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	[22]
M. extorquens AM1 mutant strains		
R1	mutated by pHT9XbHKan, *mxaR* mutant	This study
SH11	mutated by pHT9BgHKanH	This study
SN21	mutated by pHT9BgHKanN, *mxaS* mutant	This study
SA31	mutated by pHT9BgHKanA, *mxaS* mutant	This study
C31	mutated by pCMPN5Kan2, *mxaC* mutant	This study
D11	mutated by pUC19A3S1Kan, *mxaD* mutant	This study
Plasmids		
pRK310	Tc^r IncP1	[12]
pUC18/19	Ap^r *lacZ′*	[22]
pUC4K	Ap^r Kn^r from Tn*903*	Pharmacia
pHT9	8.6-kb *Hin*dIII subclone containing *mxaFJGIR* in pRK310	This study
pDN9	4.4-kb *Hin*dIII-*Xho*I subclone containing *mxaACKLD* in pVK100	[18]
pHT9Bf	3-kb *Bam*HI suclone containing *mxaRSA* in pRK310	This study
pCM59	0.8-kb subclone containing *mxaC* in pRK310	[18]
pRK310A3S1	1.9-kb subclone containing *mxaD* in pRK310	[18]
pRK310A3S2	as above but in opposite orientation	[18]
pHT9XbH19	3-kb *Xba*I-*Hin*dIII subclone in pUC19	This study
pHT9XbHKan	Kan^r inserted in *Bgl*II site in pHT9XbH19	This study
pHT9BgH18	1.4-kb *Bgl*II-*Hin*dIII subclone in pUC18	This study
pHT9BgHKanH	Kan^r inserted in *Hin*cII site in pHT9BgH18	This study
pHT9BgHKanN	Kan^r inserted in *Nru*I site in pHT9BgH18	This study
pHT9BgHKanA	Kan^r inserted in *Asc*I site in pHT9BgH18	This study
pCMPN5	2.1-kb *Pst*I-*Nru*I subclone containing *mxaC* in pUC19	[18]
pCMPN5Kan2	Kan^r inserted in *Bgl*II site in pCMPN5	This study
pUC19A3S1	1.9-kb fragment containing *mxaD* in pUC19	[18]
pUC19A3S1Kan	Kan^r inserted in *Bsa*BI site in pUC19A3S1	This study

3. Results and discussion

3.1. Electroporation of pRK310 into M. extorquens AM1

pRK310, a broad-host-range vector [12] known to be maintained in *M. extorquens* AM1 [3], was used to select optimal electroporation conditions. Efficiencies of 10^5–10^6 cells per μg of plasmid DNA were obtained using different conditions, which is a significantly higher electroporation efficiency than reported previously for *Methylobacterium* strains [6,7]. Optimal conditions were 2.5 kV, 400 Ω, 25 μF for 2-mm gap cuvette, or 2 kV, 200 Ω, 25 μF for 1-mm gap cuvettes, and time constants were 8.8–9.4 ms or 4.3–4.8 ms, respectively. Nutrient broth was chosen for the post-incubation medium, as it gave a higher recovery rate than minimum medium with succinate. Post-incubation for 1–24 h showed no significant difference for numbers of tetracycline-resistant colonies.

3.2. Mutation of mxaF by electroporation-mediated allelic exchange

The kanamycin resistance gene from pUC4K was inserted into the *Sal*I site in *mxaF*, the gene of the large subunit of methanol dehydrogenase (MDH) [8]. The gene fragment containing *mxaF*::Kan was subcloned into pAYC61, a suicide vector [13]. When this plasmid was transferred to *M. extorquens* AM1 by conjugation from *E. coli* S17-1, kanamycin-resistant colonies appeared at high frequency and those that were tetracycline-sensitive were defective in

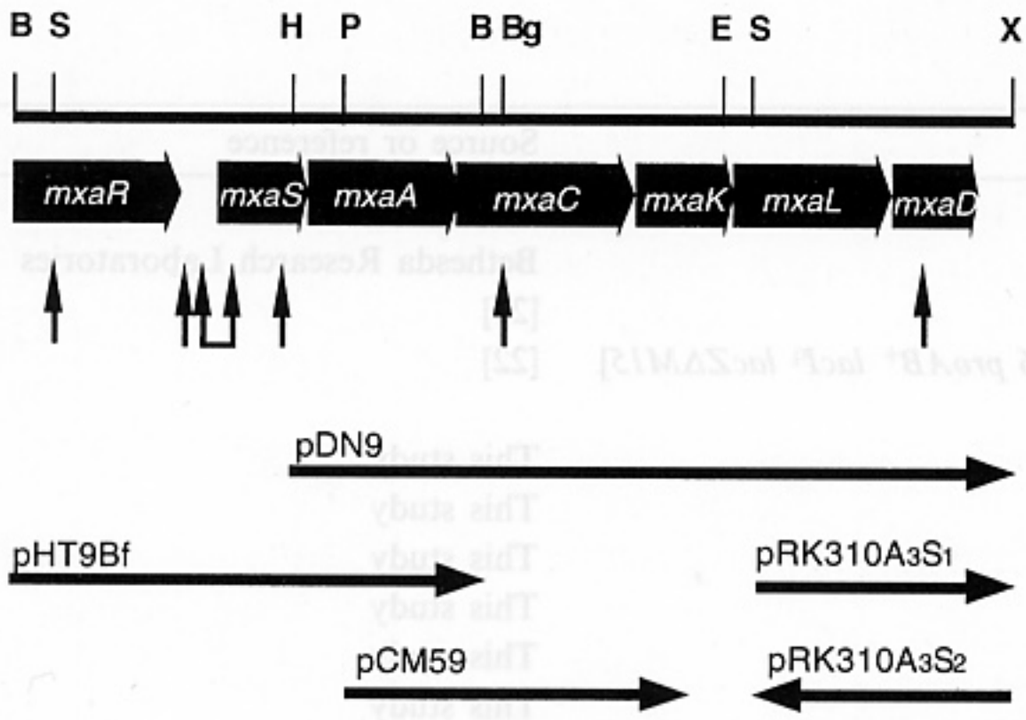


Fig. 1. Physical map of the *Bam*HI-*Xho*I DNA fragment. The open boxes represent open reading frames of *mxaRSACKLD*. Arrowheads indicate the restriction enzyme sites used for mutation. Arrows represent the cloned genes used for complementation analysis, whose directions indicate those of the pRK310 *lacZ* promoter in the plasmids listed above the genes. Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I.

growth on methanol as expected (data not shown). When the intact plasmid was introduced into *M. extorquens* AM1 by electroporation using the conditions described above, kanamycin-resistant colonies appeared. Efficiencies of 10^2–10^3 cells per μg of plasmid DNA were obtained. Each colony was patched onto agar medium containing kanamycin, tetracycline or ampicillin. Double-crossover mutants were identified as kanamycin-resistant and tetracycline-sensitive. All tetracycline-resistant colonies were ampicillin-resistant, suggesting that either marker could be used for screening, although it was reported that ampicillin resistance gene from pBR322 was not expressed in *M. extorquens* AM1 [14].

3.3. Mutation of other mxa genes

M. extorquens AM1 has a gene cluster involving methylotrophy functions, *mxaFJGIR(S)ACKLD* [15]. These genes are all transcribed in the same direction and may be a single transcriptional unit. Mutants have been isolated in *mxaFJGIACKL*, and these are all required for methanol oxidation. As noted above, *mxaF* encodes the large subunit of MDH, *mxaI* encodes the MDH small subunit, and *mxaG* encodes the MDH electron acceptor, cytochrome c_L. The exact function of *mxaJ* is unknown, but it may be an assembly protein [16]. *mxaAKL* genes are involved in insertion of calcium ions into MDH [17]. No mutants are currently available in *M. extorquens* AM1 for *mxaR*, *mxaS*, or *mxaD*, and the chemical mutants available in *mxaC* cannot be rescued by a plasmid with only *mxaC* [18]. Therefore,

Table 2
Complementation of mutants with plasmids

Strain	Plasmid	Genes	Growth on:	
			methanol	succinate
Wild-type	none	–	++	++
C31	none	–	–	++
	pDN9	*mxaACKLD*	++	++
	pCM59	*mxaC*	++	++
D11	none	–	+	++
	pDN9	*mxaACKLD*	++	++
	pRK310A3S1	*mxaD*	++	++
	pRK310A3S2	*mxaD*	+	++
R1	none	–	–	+
	pHT9	*mxaFJGIR*	+	++
	pHT9Bf	*mxaRSA*	++	++
SH11	none	–	++	++
SN21	none	–	–	++
	pHT9	*mxaFJGIR*	++	++
	pHT9Bf	*mxaRSA*	++	++
SA31	none	–	–	++
	pHT9	*mxaFJGIR*	++	++
	pHT9Bf	*mxaRSA*	++	++

```
CGTGCCCGACCTGATCCGCGCGGTGTTCGAGACGGTGCCCGCCCCGTGAGCGGTCCGGAG  1140
 V  P  D  L  I  R  A  V  F  E  T  V  P  A  P  *

      HincII
GAGGGGGTCAACGACGGGGCCGACATCATCTACCTCCCGCGCTGGCGGCCGGGCGGGCAC  1200

                                                    NruI
AGGGTCGGCGCCCATCGCGGGCCGGATGCCGGCGGTCTCGGCACGTTTCGCGATCAGGTC  1260

AGCTTCCTGCGCCTGCCGGATGGCGGGATCGACGTGCGCGCGTGCTGCGCGACCCGTTCG  1320

AGGGCGTGTTCGTGCGCGCTTCGAGGCGGCAGCCGGTCGAGACCTACGCGCTGGTCGATC  1380

TCTCCGCGTCCATGGCTTCCGCGGCCGGGCCGACCGGCGCGAACTGGCGGCCGGGTTCTG  1440
                  M  A  S  A  A  G  P  T  G  A  N  W  R  P  G  S  A

                NruI
CACCACGCTCGCCCGCTCGCGACGCGGATCGGCGACGGCTTCGGCCTGATCGCCTGCGAC  1500
  P  R  S  P  A  R  D  A  D  R  R  R  L  R  P  D  R  L  R  R

GACACTCTGCGCGACGACCTCACCCTCCCCGCGACCCGCCACCGGGCCGCCGCCCAGGCC  1560
  H  S  A  R  R  P  H  P  P  R  D  P  P  P  G  R  R  P  G  R

GCCGCCGCGCGCCTCGGCGAGGCGGCCTGCGACGGACGCGGCGCCGAGGGGCTGCTGGAG  1620
  R  R  A  P  R  R  G  G  L  R  R  T  R  R  R  G  A  A  G  A

   AscI
CGGCGCGCCGCCTTGCCGGGCGCCCGAAGCTGATCTTCCTCGTCTCGGATTTCCGCTGGC  1680
  A  R  R  L  A  G  R  P  K  L  I  F  L  V  S  D  F  R  W  P

CGGAGGCGCTGATCGAGGCGGTGTTCTCGGCGCTGGCGCTGCACGATGTGACCCCGGTGC  1740
  E  A  L  I  E  A  V  F  S  A  L  A  L  H  D  V  T  P  V  L
```

Fig. 2. Nucleotide sequence of the intergenic region between *mxaR* and *mxaS*. Amino acid sequences of the C-terminal part of MxaR and N-terminal part of the putative MxaS are shown. Restriction enzyme sites used for mutation are underlined. Accession number in the EMBL database is Y07864. Numbers at the right edge of the sequence are the same as those in [13], which start at the *Bam*HI site in Fig. 1.

insertion mutants were generated in these four genes using the electroporation procedure described for *mxaF*, except that pUC18- or pUC19-based plasmids were used instead of pAYC61 and the plasmids were linearized (Table 1, Fig. 1). The direction of the inserted drug resistance gene was determined by restriction enzyme digestion, and in each case a plasmid was chosen that had the kanamycin resistance gene in the same orientation as that of transcription of the gene of interest. In past work with insertion mutations in *M. extorquens* AM1, this orientation avoids polarity on neighboring genes [3–5]. Each plasmid was linearized with an appropriate restriction enzyme, precipitated by ethanol, dissolved in deionized water, and then used for electroporation. Recombinants were selected on agar plates containing kanamycin and screened for ampicillin resistance. Efficiencies were around 10^2–10^3 cells per μg of DNA. Most of the colonies obtained on kanamycin were ampicillin-sensitive. Double-crossover mutations were confirmed by DNA hybridization with digoxigenin-labelled pUC18 or the kanamycin resistance gene from pUC4K as a probe.

The *mxaC* insertion mutant, C31, did not grow on methanol (Table 2). Strain C31 can be complemented by either pDN9 or pCM59, both of which have *mxaC*. The insertion mutant of *mxaD* (D11) grew less well than the wild-type did on methanol agar plates, while it grew as well as the wild-type on succinate or methylamine plates. The reduced growth on methanol was recovered by pRK310A3S1 but not pRK310A3S2, which contain *mxaD* in opposite orientations with respect to the *lac* promoter in pRK310 with pRK310A3S1 in the correct orien-

tation (Fig. 1) [18]. These results suggest that *mxaD* is involved in methanol utilization but is not essential to growth on methanol.

Between *mxaFJGI* and *mxaACKLD*, two probable open reading frames, *mxaRS*, are present that show identity to previously known open reading frames required for methanol oxidation in *Paracoccus denitrificans* [15]. However, in *M. extorquens* AM1 *mxaS* has atypical codon usage at the start of the putative open reading frame and this portion of the putative *mxaS* does not show identity to the *P. denitrificans* *mxaS*. Therefore, it is not known whether *mxaS* encodes a gene product in *M. extorquens* AM1 [15]. Three constructions were used to analyze the *mxaS* region by mutation (Fig. 1). One has the kanamycin resistance gene inserted in a *Hin*cII site existing in the probable intergenic region between *mxaR* and *mxaS*. The mutant obtained in this case (SH11) was able to grow on methanol as well as wild-type, suggesting that it interrupted a non-coding region and that the inserted gene had little or no polar effect on neighboring genes. The insertion mutant obtained at the same position but in the opposite orientation also grew on methanol but less well than the wild-type, suggesting a mild polar effect. A second mutant (SN21) contains a deletion of a 202-bp *Nru*I fragment, which removes about half of the probable intergenic region and deletes the first 23 amino acids of MxaS (Fig. 2). This *mxaS* deletion is within the region that does not show similarity to *mxaS* of *P. denitrificans* (Fig. 2). Mutant SN21 did not grow on methanol (Table 2). A third mutant (SA31) was obtained by inserting the kanamycin resistance gene in the *Asc*I site present in the middle of the portion of *mxaS* that shows identity to *mxaS* of *P. denitrificans*. Strain SA31 showed no growth on methanol either. A plasmid containing the *mxaRSA* region (pHT9Bf) complemented the growth of the latter two mutants on methanol. These results suggest that *mxaS* is translated and its product is involved in methanol oxidation, even though it contains atypical codon usage at the start of the open reading frame.

The *mxaR* gene is also a gene of unknown function. Sequence analysis has shown that the *mxaR* gene product is similar to MoxR of *P. denitrificans* and has predicted MgATP binding motifs A and B [15]. An *mxaR* insertion mutant (R1) was obtained and it did not grow on methanol. The mutant grew on succinate less well than wild-type (Table 2). Growth on methanol was restored by pHT9Bf, which contains *mxaRSA*. Although the function of the *mxaR* gene product is still unknown, apparent homologs of this gene have been found in the genome sequences of *Bacillus subtilis* [19] and *Archeoglobus fulgidus* [20], and so it probably represents a new gene family of widespread distribution.

In this article, we have shown that gene disruption by homologous recombination in *M. extorquens* AM1 is possible by electroporation of linear fragments. This method facilitates the generation of mutants in genes that have been cloned and sequenced.

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