

# Phage Mu-driven two-plasmid system for integration of recombinant DNA in the *Methylophilus methylotrophus* genome

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Received: 10 July 2008 / Revised: 27 August 2008 / Accepted: 1 September 2008 / Published online: 27 September 2008  
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**Abstract** A phage Mu-driven two-plasmid system for DNA integration in *Escherichia coli* genome has been adjusted for *Methylophilus methylotrophus*. Constructed helper plasmids with broad-host-range replicons carry thermo-inducible genes for transposition factors MuA and MuB. Integrative plasmids that are only replicated in *E. coli* could be mobilized to *M. methylotrophus* and contained mini-Mu unit with a short terminus of Mu DNA, Mu-*attL/R*. Mini-Mu unit was integrated in the *M. methylotrophus* genome via mobilization of the integrative plasmid to the cells carrying the helper in conditions of thermo-induced expression of MuA and MuB. In this system, mini-Mu unit was mainly integrated due to replicative transposition, and the integrated copy could be amplified in the *M. methylotrophus* chromosome in the presence of helper plasmid. A *kan*-gene flanked by *FRT* sites was inserted in one of the mini-Mu units, and it could be readily excised by yeast FLP recombinase that is encoded by the designed plasmid. The multiple Mu-driven gene insertion was carried out by integration of the *Bacillus amyloliquefaciens*  $\alpha$ -amylase gene followed by curing the Km<sup>R</sup> marker before integration of the second mini-Mu unit with *Pseudomonas putida xylE* gene encoding catechol 2,3-dioxygenase (C23O).

**Keywords** Methylotrophs · Mini-Mu unit · Replicative transposition · Amplification · Excisable marker

## Introduction

Methylotrophs are a diverse group of organisms that are capable of growing on single-carbon substrates, such as methane or methanol, as sole sources of organic carbon and energy. The availability of the raw material and its relatively low price make methylotrophic bacteria interesting candidates as producers of numerous biologically active compounds. Therefore, these microorganisms are currently becoming more and more widely used in the manufacturing of natural and recombinant proteins (Bélanger et al. 2004; Fitzgerald and Lidstrom 2003), polysaccharides, amino acids, and vitamins (Bourque et al. 1995; Korotkova et al. 2002; Motoyama et al. 1993, 2001).

The industrial application of methylotrophs has focused attention on the investigation of genetic regulation of C-1 metabolism (Anthony 1993). However, the majority of these bacteria have been poorly studied, and the genetic tools required for their investigation are limited. The *Methylophilus methylotrophus* that is the subject of our investigation is especially hard to manipulate genetically because the majority of amino acids fail to penetrate in and export out of its cells, and therefore, the conventional methods for obtaining auxotrophic mutants are inapplicable under the circumstances. On the other hand, existing data on efficient expression of a few *Escherichia coli* genes in

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*M. methylotrophus* (see for example Gunji and Yasueda 2006; Tsujimoto et al. 2006) permit the hope of practical application of well-developed genetic systems to these methylotrophs.

Along with design of plasmid vectors, improvement of transposon mutagenesis is one of the priority genetic techniques for cloning and gene expression in methylotrophic bacteria (Koch et al. 2001; Marx and Lidstrom 2001, 2004). For example, a possibility of using Tn7 for site-specific integration of recombinant DNA in the genome of *Methylobacterium extorquens* was demonstrated (Choi et al. 2006). The system can be applied to integration in a unique site in the recipient bacterial genome (Koch et al. 2001). In order to allow multiple integration of several copies of gene of interest and/or many dissimilar genes in different points of bacterial genome, the use of a well-investigated, both in vivo and in vitro, mobile genetic element, bacteriophage-transposon Mu (Mizuuchi 1983), seemed most promising.

It is known that Mu employs two transposition mechanisms depending on the stage of the vital cycle: (1) simple insertion upon infection and introduction of its linear DNA, practically, into the random point of the bacterial genome and (2) replicative transposition via formation of cointegrates at the lytic development and amplification of phage DNA (Craigie and Mizuuchi 1985; Sokolsky and Baker 2003).

Although some undetermined details that are being intensely investigated (see for example Yin et al. 2007) remain in the mechanism of Mu transposition, efficient systems for integration of recombinant DNA in vitro (Haapa et al. 1999) and in vivo in *E. coli* (Akhverdyan et al. 2007; Groisman and Casadaban 1987) and *Salmonella typhimurium* (Lawes and Maloy 1995) chromosomes have already been designed on its basis and are rather actively being used. In the present study, we have shown that it is also possible to perform the Mu-driven integration of recombinant DNA in the chromosome of *M. methylotrophus*. To this end, first of all, helper plasmids with thermo-inducible genes encoding transposition factors of phage Mu (MuA and MuB) were constructed on the basis of broad-host-range replicons of IncP and IncQ incompatibility groups. Integrative vectors, plasmids with constructed mini-Mu units that are capable of mobilization transfer to *M. methylotrophus* cells, were also designed. Mini-Mu units from the integrative vectors flanked by Mu-*attL* and Mu-*attR* DNA fragments were transposed in the chromosome of *M. methylotrophus* during the induced synthesis of MuA and MuB. Mini-Mu unit contained kanamycin resistance gene (*kan*) flanked with *FRT* sites that permitted the selection of integrants for their resistance to kanamycin (Km) and then to eliminate the marker from the bacterial chromosome exploiting the *Saccharomyces cerevisiae*

FLP-*FRT*-mediated site-specific recombination system (Senecoff et al. 1985) adjusted to expression in *M. methylotrophus*.

## Materials and methods

### Bacterial strains, plasmids, and cultivation conditions

Strains and plasmids used in the study are shown in Table 1. Cells of *M. methylotrophus* AS1 were grown at 37°C on a mineral medium SEIIa (Gunji et al. 2004) of the following composition: K<sub>2</sub>HPO<sub>4</sub>, 1.9 g; NaH<sub>2</sub>PO<sub>4</sub>×2H<sub>2</sub>O, 1.56 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 g; MgSO<sub>4</sub>×7H<sub>2</sub>O, 200 mg; CaCl<sub>2</sub>×2H<sub>2</sub>O, 72 mg; CuSO<sub>4</sub>×5H<sub>2</sub>O, 5 µg; MnSO<sub>4</sub>×5H<sub>2</sub>O, 25 µg; ZnSO<sub>4</sub>×7H<sub>2</sub>O, 23 µg; FeCl<sub>3</sub>×6H<sub>2</sub>O, 9.7 mg/l, methanol 2%, pH 7.0. Bactoagar (1.2%, Difco, USA) and methanol 1% were applied to the solid media. *M. methylotrophus* AS1 was resistant to chloramphenicol (Cm), thus permitting the employment of this antibiotic to the counter-selection of donor *E. coli* S17-1-based strains in bacterial mating experiments (see below). Antibiotics to maintain plasmid DNA in *M. methylotrophus* were added at the following concentrations: ampicillin (Ap), 100 µg/ml; tetracycline (Tc), 2 µg/ml; and streptomycin (Sm), 50 µg/ml. Km (10 µg/ml) and Cm (20 µg/ml) were used for selection of the integrants and counter-selection of the donor, respectively. Selection of clones containing the amplified mini-Mu {[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>} unit in the *M. methylotrophus* chromosome was performed on agar medium SEIIa with the Sm content of 2 mg/ml. The *E. coli* strains were cultured at 37°C on liquid or solid Luria–Berthani (LB) medium; 1.2% of bactoagar was added in the latter case (Sambrook and Russell 2001). The antibiotics (Ap, 200 µg/ml; Tc, 20 µg/ml; and Sm, 50 µg/ml) were added during growth of the appropriate *E. coli* plasmid strains. All manipulations with strains *M. methylotrophus* AS1 and *E. coli* with plasmids containing thermo-inducible genes, MuA, MuB, or FLP, were carried out at 30°C unless induction was not required. Selection of the in vitro constructed recombinant plasmids was conducted in the strains *E. coli* TG1 and *E. coli* S17-1.

### Standard gene engineering methods

The treatment of recombinant DNA and the Southern hybridization was carried out in accordance to conventional protocols (Sambrook and Russell 2001). Preparations of restrictases, T4 DNA ligases, and DNA polymerase I Klenow fragment from Fermentas (Lithuania) were used. Taq DNA polymerase (Fermentas) or AccuTaqLA DNA polymerase (Sigma) were used in accordance with the manufacturer's instructions to provide polymerase chain

**Table 1** Strains and plasmids used in this study

Strain and plasmid	Relevant characteristics	Reference or source
<b>Strains</b>		
<i>M. methylotrophus</i> AS-1	Wild type	NCIMB10515
<i>E. coli</i>		
TG1	(F <sup>-</sup> ) Δ(lac-pro) supE thi hsdΔ5/F' traD36 proA <sup>+</sup> B <sup>+</sup> lacI <sup>q</sup> lacZΔM15	VKM IMG-341
S17-1	Tp <sup>R</sup> ; Sm <sup>R</sup> ; (F <sup>-</sup> ) RecA pro thi hsdR <sup>-</sup> hsdM <sup>+</sup> RP4-2-Tc::Mu-Km::Tn7	ATCC47055
<b>Plasmids</b>		
pRK310	Tc <sup>R</sup> ; IncPα Tra <sup>-</sup> broad-host-range cloning vector derived from RP4	Ditta et al. 1985
pAYC32	Ap <sup>R</sup> ; Sm <sup>R</sup> ; IncQ broad-host-range cloning vector derived from RSF1010::Tn1	Chistorerdov and Tsygankov 1986
pAYCTER3	Ap <sup>R</sup> ; IncQ broad-host-range cloning vector derived from pAYC32	Gulevich et al. 2006
pUC-MuAB	Ap <sup>R</sup> ; pUC1918 with the 5.7 kb <i>ScaI</i> – <i>Eco</i> 47III fragment from pMud4041 containing the <i>MuAB ner c<sup>ts</sup></i> genes	Akhverdyan et al. 2007
pSUP5011	Ap <sup>R</sup> ; Cm <sup>R</sup> ; Neo <sup>R</sup> ; pBR325(Bam <sup>-</sup> ):Tn5- <i>Mob<sup>+</sup></i> <sub>RP4</sub>	Simon et al. 1983, 1984
pKD4	Km <sup>R</sup> ; Ap <sup>R</sup> ; pANTSγ[FRT-Km <sup>R</sup> -FRT]	Datsenko and Wanner 2000
pCP20	Cm <sup>R</sup> ; Ap <sup>R</sup> ; plasmid with <i>λ</i> I <sub>857</sub> <sup>ts</sup> <i>lcro-FLP</i>	Datsenko and Wanner 2000
pUX1918	Ap <sup>R</sup> ; pUC1918- <i>XylE</i>	Schweizer 1993
pRT5	pSM19035 deletion derivative containing the <i>B. amyloliquifaciens amyE</i> gene	Smirnova et al. 1988
pHP17	Ap <sup>R</sup> ; Sm <sup>R</sup> pAYCTER3 with the <i>M. methylotrophus</i> promoter P17	Tokmakova, unpublished
pMIV5	Ap <sup>R</sup> ; pMW119 with mini-Mu containing MCS <sub>pUC59</sub> flanked with transcription terminators <i>thrL</i> and <i>2fd</i> between Mu- <i>attL/R</i>	Tokmakova et al. 2007
pMIV5-Mob	Ap <sup>R</sup> ; pMIV5 derivative containing the <i>Bam</i> HI fragment from pSUP5011, including <i>Mob<sup>+</sup></i> <sub>RP4</sub>	This study
pMIV5-Mob-[FRT-Km <sup>R</sup> -FRT]	Ap <sup>R</sup> ; Km <sup>R</sup> ; pMIV5-Mob with the 1.5 kb <i>Hind</i> III– <i>Nde</i> I fragment from pKD4 containing the Km <sup>R</sup> gene flanked <i>FRT</i> sites	This study
pMIV5-[FRT-Km <sup>R</sup> -FRT]- <i>amy</i> -Mob;	Ap <sup>R</sup> ; Km <sup>R</sup> ; pMIV5-Mob[FRT-Km <sup>R</sup> -FRT] with the 2.9 kb <i>Bgl</i> II– <i>Bam</i> HI fragment from pRT5 containing the <i>B. amyloliquifaciens amyE</i> gene	This study
pMIV5-[FRT-Km <sup>R</sup> -FRT]- <i>xyl</i> -Mob;	Ap <sup>R</sup> ; Km <sup>R</sup> ; pMIV5-Mob-[FRT-Km <sup>R</sup> -FRT] with the <i>Eco</i> RI fragment from pX1918 containing the <i>P. putida xylE</i> gene	This study
pMIV5-[FRT-Km <sup>R</sup> -FRT]-Sm <sup>R</sup> -Mob;	Ap <sup>R</sup> ; Km <sup>R</sup> ; Sm <sup>R</sup> ; pMIV5-Mob[FRT-Km <sup>R</sup> -FRT] with the 1.9 kb <i>Eco</i> RV fragment containing the <i>strAB</i> genes under control the <i>M. methylotrophus</i> promoter P17	This study
pTP310	Tc <sup>R</sup> ; pRK310 with the 5.7 kb <i>Bam</i> HI fragment from pUC-MuAB containing the <i>MuAB ner c<sup>ts</sup></i> genes	This study
pAET7	Ap <sup>R</sup> ; Sm <sup>R</sup> ; pAYC32 with the 5.7 kb <i>Bam</i> HI fragment from pUC-MuAB containing the <i>MuAB ner c<sup>ts</sup></i> genes	This study
pFLP31	Ap <sup>R</sup> ; Sm <sup>R</sup> ; pAYCTER3 with the 3.3 kb <i>Sma</i> I– <i>Bam</i> HI fragment from pCP20 containing the <i>λ</i> I <sub>857</sub> <sup>ts</sup> <i>lcro-FLP</i> genes	This study

reaction (PCR) for confirmation the chromosomal modifications. The primers P1:5'-ttagatttggtgggcttgc→3'; P2:5'-gtttatcagcttgccttcgagg→3' were used. The Biotin Deca-Label™ kit and biotin chromogenic detection kits (Fermentas) were used to label and detect DNA probes in Southern hybridization.

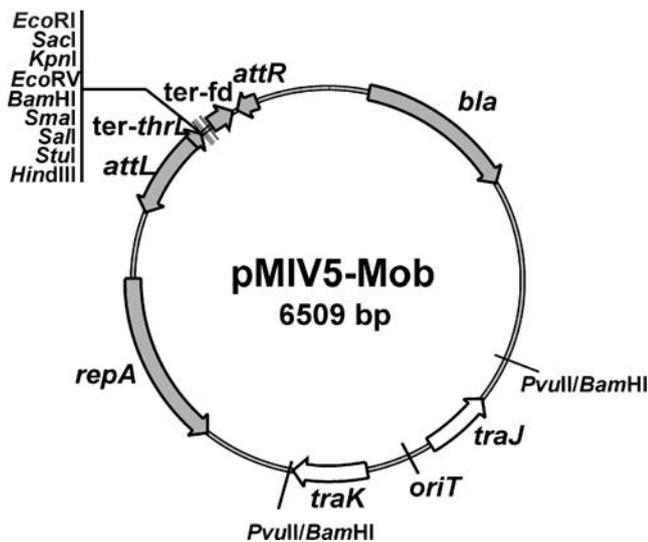
#### Construction of recombinant plasmids

pAET7 (Ap<sup>R</sup>, Sm<sup>R</sup>) was obtained on the basis of pAYC32 (Chistorerdov and Tsygankov 1986) by cloning of *Bam*HI fragment from pUC-MuAB (Akhverdyan et al. 2007).

pTP310 (Tc<sup>R</sup>) was a result of cloning the same *Bam*HI fragment of pUC-MuAB in broad-host-range replicon

pRK310, a derivative of plasmid RP4/RK2 of IncPα group (Ditta et al. 1985; Pansegrau et al. 1994).

pMIV5 (Tokmakova et al. 2007) containing a multiple-cloning site (MCS) flanked with transcription terminators between Mu-*attL* and Mu-*attR* was used as a vector for construction of the integrative plasmids. In order to obtain pMIV5-Mob, the blunted *Bam*HI fragment (~1.9 kb) of pSUP5011 (Simon et al. 1983, 1984) containing *oriT*, *traJ*, and *traK* from plasmid RP4 (Pansegrau et al. 1990) was cloned into the *Pvu*II site of pMIV5 located outside of mini-Mu unit in the region that is nonessential for replication (Fig. 1). After that, the blunted 1.5 kb *Hind*III–*Nde*I fragment of pKD4 (Datsenko and Wanner 2000) containing *kan* flanked with *FRT* sites was cloned into



**Fig. 1** Map of the vector plasmid pMIV5-Mob. *ter-thrL* transcription terminator of *E. coli thrL* gene, *ter-fd*, a tandem transcription terminator of phage fd

*EcoRV* site of pMIV5-Mob to produce pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob (Fig. 2).

Three integrative plasmids were constructed using pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob as a vector:

- The blunted 2.9-kb *BglII*–*BamHI* fragment of pRT5 (Smirnova et al. 1988) containing the *amyE* gene for  $\alpha$ -amylase of *B. amyloliquefaciens* was inserted into the *SmaI* site of the vector to obtain pMIV5-[FRT-Km<sup>R</sup>-FRT]-*amy*-Mob;
- The *EcoRI* DNA fragment of pX1918 (Schweizer 1993) containing the structural part of the *xylE* gene of the TOL plasmid of *P. putida* mt-2 (Inouye et al. 1981) was inserted into the *EcoRI* site of the vector to put the cloned gene under the transcriptional control of Km<sup>R</sup> and create pMIV5-[FRT-Km<sup>R</sup>-FRT]-*xyl*-Mob;
- The 1.9-kb *EcoRV* fragment of pHP17 (Tokmakova, unpublished) containing *strAB* under control of a weak promoter P17 of *M. methylotrophus* was inserted into a

*SmaI* site of the vector DNA to make pMIV5-[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>-Mob (Fig. 2).

The FLP recombinase expressing plasmid pFLP31 was constructed on the basis of pAYCTER3 (Gulevich et al. 2006) derived from pAYC32 (Chistorerdov and Tsygankov 1986) by cloning the *BamHI*–*SmaI* fragment (3.3 kb) of pCP20 DNA (Datsenko and Wanner 2000) containing the gene *FLP* of *S. cerevisiae* that was transcribed from promoter  $\lambda$ P<sub>R</sub> governed by the plasmid encoded  $\lambda$ CI<sub>ts857</sub>. The pFLP31 can be eliminated from more than 30% of cells of *M. methylotrophus* after growth for 20 generations at 30°C without antibiotic on SEII medium containing 25–30  $\mu$ g/ml of acridine orange.

#### Bacterial mating

Integrative and helper plasmids were transferred into strain *M. methylotrophus* AS1 by biparental mating using *E. coli* S17-1 bearing the respective plasmid as the donor. The overnight donor and recipient cultures ( $5 \times 10^8$ – $10^9$  cell/ml) were mixed at the ratio 1:10, harvested by centrifugation, washed with 0.15 M NaCl, and placed on plates with the SEIIa medium containing 10% of LB broth and 0.5% of methanol. After 3–4 h of incubation at 30°C, the conjugation mixture was washed off from the plates with 1 ml of 0.15 M NaCl and seeded in a dilution on agar SEIIa medium that contained the appropriate antibiotic (Ap, Tc, or Sm) for selection of *M. methylotrophus* plasmid-carrier variants and Cm for donor *E. coli* strain counter-selection.

#### Integration of mini-Mu unit in the chromosome

Transposition of mini-Mu unit into the *M. methylotrophus* chromosome was performed by conjugative transfer of integrative plasmid to the recipient strain *M. methylotrophus* that contained a helper plasmid. Conjugation was carried out at 37°C to induce the synthesis of the transposition factors. The Km<sup>R</sup> integrants were selected

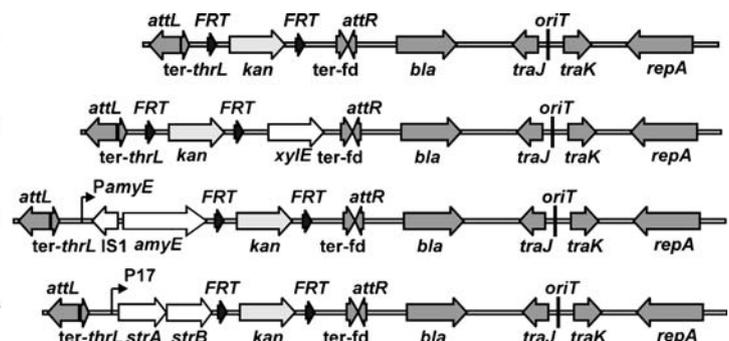
**Fig. 2** Linear representation of the integrative plasmids derived from pMIV5-Mob

pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob (8028 bp)

pMIV5-[FRT-Km<sup>R</sup>-FRT]-*xyl*-Mob (9465 bp)

pMIV5-[FRT-Km<sup>R</sup>-FRT]-*amy*-Mob (10937 bp)

pMIV5-[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>-Mob (9895 bp)



on solid SEIIa medium containing Km and Cm for donor counter-selection.

Elimination of [FRT-Km<sup>R</sup>-FRT] marker from the chromosome

pFLP31 was transferred into strain *M. methylotrophus* containing the integrated mini-Mu unit with [FRT-Km<sup>R</sup>-FRT] marker by mobilization (using plasmid-encoded Ap<sup>R</sup> as the selective marker), and the Km<sup>R</sup>Ap<sup>R</sup> colonies were isolated. The isolated colonies were suspended up to the final concentration of 10<sup>7</sup> cell/ml in 5 ml of liquid medium SEIIa with Ap, and the suspension was heated at 42°C for 20 min with the following incubation on a shaker at 37°C for 16–18 h to induce the FLP recombinase synthesis. The culture was then plated on the non-selective SEIIa medium to obtain individual colonies. The latter were analyzed for the occurrence of the Km and Ap markers on the solid SEIIa medium with the appropriate antibiotic.

Detection of activities of  $\alpha$ -amylase and C23O in recombinant *M. methylotrophus* strains

A modified method of insoluble starch (amylopectin azure) hydrolysis was used for the qualitative analysis of  $\alpha$ -amylase activity (Mantsala and Zalkin 1979). The replicas of the *M. methylotrophus*::{[FRT-Km<sup>R</sup>-FRT]-amy} integrants were applied to Petri dishes containing the solid SEIIa medium (1.5% amylopectin azure) and incubated at 37°C. Visual detection of starch hydrolysis zones that correlated with  $\alpha$ -amylase-active colonies was performed on the third and fourth day.

The activity of catechol-2,3-dioxygenase (C23O) in the *M. methylotrophus*::{[FRT-Km<sup>R</sup>-FRT]-xyIE} cells was determined by the appearance of bright yellow color after colonies were sprayed with a solution of catechol (100 mM; Zukowski et al. 1983).

Amplification of mini-Mu unit in the chromosome of *M. methylotrophus*

pTP310 was conjugatively transferred into strain *M. methylotrophus*::{[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>} using Tc<sup>R</sup> as a marker. After overnight incubation at 37°C on a shaker cell cultures were plated on the agar SEIIa medium containing different amounts of Sm, and clones resistant to the high concentration of Sm (2.0 mg/ml) were selected. The helper plasmid was eliminated from the selected clones during aerobic cultivation in the liquid SEIIa medium at 30°C for 24 h. The presence of additional copies of mini-Mu unit in the chromosomal DNA of *M. methylotrophus* resistant to high concentration of Sm was confirmed by Southern DNA hybridization.

## Results

Adaptation of the two-plasmid system of Mu-driven transposition to investigations in *M. methylotrophus*

The helper plasmid pUC-MuAB was constructed and used earlier as a component of the two-plasmid system for Mu transposition in *E. coli* (Akhverdyan et al. 2007). A gene for the temperature-sensitive repressor, *cts62*, and the genes for transposition factors MuA and MuB were located in this plasmid. However, being constructed on the pUC-like replicon, pUC-MuAB could only be replicated and maintained in *E. coli* cells. Therefore, a modification of the helper plasmid was necessary in order to use the system in *M. methylotrophus*.

Two plasmids were constructed on the basis of broad-host-range replicons of different groups of incompatibility and were capable of being transferred to the methylotrophs by mobilization. One of them, pAET7 (Ap<sup>R</sup>, Sm<sup>R</sup>, IncQ group; Guerry et al. 1974), was stably maintained in *M. methylotrophus* (percentage of cells that lost the plasmid within 20 generations under non-selective conditions at 30°C was no greater than 2%). pAET7 could be eliminated with the frequency of no less than 30% by growing the *M. methylotrophus* in a medium with acridine orange (25–30  $\mu$ g/ml) for 24–48 h. The second helper plasmid, pTP310 (Tc<sup>R</sup>, IncP $\alpha$ -group; Ditta et al. 1985; Pansegrau et al. 1994), was maintained in the *M. methylotrophus* only under strictly selective conditions and qualitatively lost from the population when aerobically cultured (30°C, 48 h) in liquid medium without Tc.

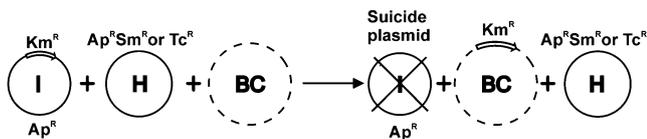
The second component of two-plasmid system for Mu-driven transposition is integrative vector. To increase the efficiency of vector transfer into variety of microorganisms, the fragment containing the RP4-specific *mob* site was cloned into pMIV5 containing Mu-*attL/R* sites. Then, Km<sup>R</sup> flanked by the *FRT* sites was introduced in mini-Mu unit as a marker to give pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob (see “Materials and methods”; Figs. 1 and 2).

The obtained pAET7 and pTP310 can be used as helper plasmids, and pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob, in turn, as a mobilizable integrative plasmid with a selective marker, in the testing of Mu-driven integration in *M. methylotrophus* cells.

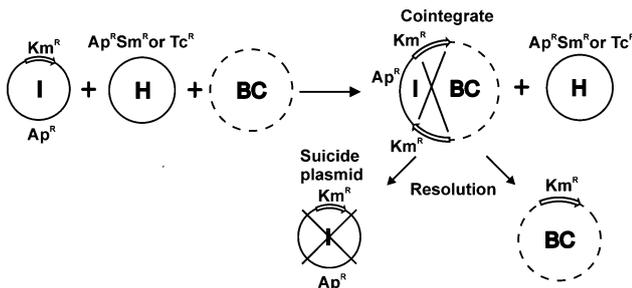
Mu-driven transposition of Km<sup>R</sup> in the *M. methylotrophus* chromosome and FLP-mediated curing the marker

The helper and integrative plasmids were applied to transformation of *E. coli* S17-1 (Tra<sup>+</sup>), and the obtained strains were used in succession as donors in the conjugation crossings with *M. methylotrophus* AS1. This methylotrophic strain was resistant to Cm, thus permitting the

## a. Simple insertion



## b. Replicative transposition



**Fig. 3** Simple insertion or replicative transposition of  $Km^R$ -mini-Mu unit (an arrow) located in the only replicated in *E. coli* integrative  $Ap^R$  plasmid (I) in bacterial chromosome (BC) of *M. methylotrophus* in the presence of different helper plasmids (H) carrying  $Ap^R$  and  $Sm^R$  or  $Tc^R$  genes. Transposition of the mini-Mu unit from I can result in a simple insertion in which BC obtains a copy of this unit (a) or a cointegrate in which I and BC fuse and two copies of the mini-Mu unit border the junction of the fused replicons as direct repeats (b). The cointegrate can subsequently be resolved by a reciprocal recombination event between two mini-Mu units

employment of this antibiotic to the donor counter-selection. At the first stage, the helper plasmid pAET7 was introduced in *M. methylotrophus* (with the efficiency  $10^{-2}$ – $10^{-3}$  relative to the initial number of the donor strain cells) by mobilization using  $Sm^R$  marker. At the second stage, *M. methylotrophus* AS1 that contains a helper plasmid was used as recipient in crossing with *E. coli* S17-1/pMIV5-[FRT- $Km^R$ -FRT]-Mob. Mobilization was performed at 37°C, ensuring the thermo-induced expression of MuA and MuB. The  $Km^R$  integrants were selected with the frequency of  $10^{-2}$ – $10^{-3}$  that correlated with the efficiency of plasmid mobilization, i.e., transposition of  $Km^R$  marker occurs practically in each cell of the recipient strain that received the integrative plasmid. Later on, after

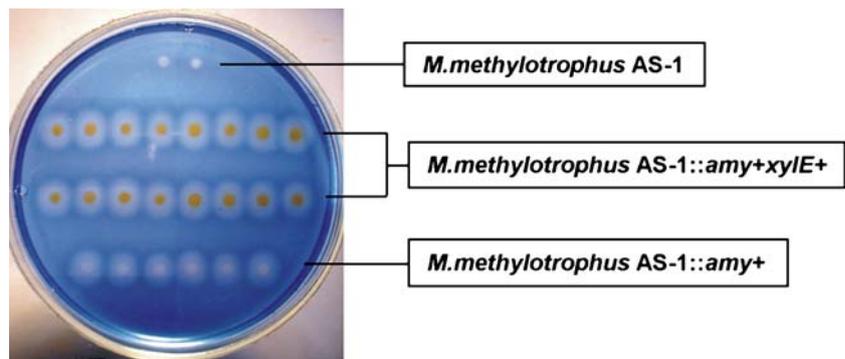
pAET7 was cured in the  $Km^R$  cells, the occurrence of mini-Mu unit in the bacterial chromosome was confirmed by genetic analysis ( $Ap^S$ ) and PCR (using the mentioned primers P1 and P2 in “Materials and methods,” we detected the DNA fragment of about 2,200 bp that corresponded to the size between Mu-*attL/R* sites in the used  $Km^R$ -mini-Mu unit).

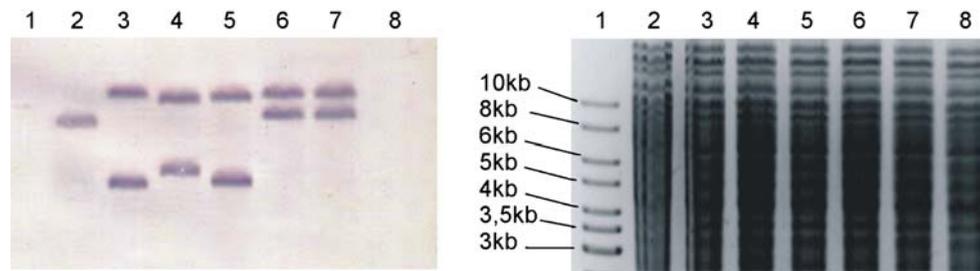
Since both integrative and helper plasmids had the same  $Ap^R$  marker, the  $Ap^R K m^R$  cointegrates that could be formed in the process of replicative transposition would be genetically undistinguishable from the  $Km^R$  integrants obtained due to the simple insertion and containing the  $Ap^R$  helper plasmid (see Fig. 3). During the long procedure required for the pAET7 elimination, the cointegrates could have resolved, giving the same  $Km^R$  phenotype, as the products of simple insertion lost the helper plasmid. Therefore, using pAET7 as the helper plasmid, it was impossible to estimate which of the known mechanisms was involved in the mini-Mu unit transposition into the genome of *M. methylotrophus*.

An attempt to detect the cointegrates was carried out using another helper, pTP310, that did not contain  $Ap^R$  and carried  $Tc^R$  as a selective marker. After mobilization of pMIV5-[FRT- $Km^R$ -FRT]-Mob into *M. methylotrophus* AS1/pTP310 and selection of  $Km^R$  methylotrophic clones, they were examined for the Ap resistance. Only 30% of these clones had phenotype  $Km^R, Ap^S$ , i.e., they were resulted from either the simple insertion or replicative transposition event, having the cointegrates already resolved. The remaining 70% of the analyzed clones had the  $Km^R, Ap^R$  phenotype being true cointegrates (Fig. 3). Further cultivation of these clones in  $Km$ -containing medium (without Ap) led to the selection of  $Km^R Ap^S$  variants that were the products of cointegrate resolution.

In this work, *kan* gene flanked with the *FRT* regions was used as the selective  $Km^R$  marker for mini-Mu unit integration. To excise this marker out of the *M. methylotrophus* chromosome, the pFLP31 plasmid bearing the FLP recombinase of *S. cerevisiae* under the control of the thermo-inducible promoter  $\lambda P_R$  was constructed. The

**Fig. 4** Visual detection of activities of  $\alpha$ -amylase (starch hydrolysis zones) and C230 (bright yellow color) in the colonies of *M. methylotrophus* integrants





**Fig. 5** Southern blot analysis of the clones of *M. methylotrophus* with integrated and amplified mini-Mu {[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>} unit. Genomic DNA, prepared from 15 colonies selected on 2.0 mg/ml of Sm and from the control strains was digested with *EcoRV*, separated by gel electrophoresis in agarose (*right*) and hybridized with the

internal fragment of *kan* used as the probe (*left*). Five of 15 samples are shown here. *Lane 1* DNA ladder, *lane 2* a control clone with non amplified mini-Mu unit, *lanes 3-7* clones with amplified mini-Mu unit, *lane 8* a negative control a wild-type clone

pFLP31 was transferred to the strain *M. methylotrophus* that contained mini-Mu unit with Km<sup>R</sup> marker in the chromosome, and FLP synthesis was thermo-induced. After culture plating, 99% of the selected Ap<sup>R</sup> clones had the Km<sup>S</sup> phenotype. The Km<sup>R</sup> marker excision was confirmed using PCR (using primers P1 and P2 in PCR we detected the decrease of the amplimers' size from about 2,200 to 815 bp after the marker excision). pFLP31 was eliminated from the obtained Km<sup>S</sup> clones. The resulting markerless strain can be used as recipient for further modifications.

Consecutive Mu-driven transposition of heterologous genes to *M. methylotrophus*

The multiple modification of *M. methylotrophus* strain was demonstrated using the consecutive integration of a few genes cloned in pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob into the *M. methylotrophus* chromosome.

Firstly, the gene for  $\alpha$ -amylase (*amyE*) of *B. amyloliquefaciens* was cloned into pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob, and the resulting mini-Mu unit was integrated into the *M. methylotrophus* chromosome using Km<sup>R</sup> as the selective marker. After the FLP-FRT-mediated Km<sup>R</sup> excision was performed, expression of *amyE* gene was detected in the obtained strain by zones of hydrolysis on a starch-containing medium (Fig. 4).

Secondly, a structural part of the *xylE* gene in the TOL plasmid of *P. putida* mt-2 was cloned into pMIV5-[FRT-Km<sup>R</sup>-FRT]-Mob under transcriptional control of the *kan* promoter. The mini-Mu unit obtained was integrated into *M. methylotrophus* AS1 and *M. methylotrophus* AS1::*amy*<sup>+</sup>. The expression of *amyE* and *xylE* in the double integrant strain was demonstrated (Fig. 4).

Mini-Mu unit amplification in the *M. methylotrophus* chromosome

Integration of mini-Mu unit in the *M. methylotrophus* chromosome occurs mainly by replicative transposition;

therefore, this mechanism can be employed for the amplification of the integrated genes. Mini-Mu unit {[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>} was used for amplification as a model. It was already known that a single copy of *strAB* under the control of P17 promoter can provide the resistance of *M. methylotrophus* to 500  $\mu$ g/ml of Sm, whereas methylotrophic cells that contained this gene on a moderate-copy-number plasmid were resistant to >2 mg/ml of Sm (Tokmakova, unpublished). In the presence of pTP310 in conditions of induced MuA and MuB synthesis, the clones containing amplified mini-Mu {[FRT-Km<sup>R</sup>-FRT]-Sm<sup>R</sup>} units were selected on the plates containing 2 mg/ml of Sm, but with rather low frequency— $2 \times 10^{-4}$ . The fact that the elevated resistance of the selected clones was due to the mini-Mu unit amplification was proven by the Southern hybridization (Fig. 5). The selected clones had at least two copies of mini-Mu unit in their genomes. Unfortunately, the presence of two mini-Mu units in the chromosome has already provided resistance to such high antibiotic concentrations (up to 10 mg/ml) that selection of variants with higher copy number was made impossible.<sup>1</sup> However, the possibility of mini-Mu unit intrachromosomal amplification in the presence of Mu transposition factors was demonstrated. It seems probable that this amplification based on the known intrachromosomal replicative transposition resulted in cointegrate formation leading to chromosomal rearrangements due to the inversion of the chromosomal fragment located between two obtained copies of transposon (Metzler 2001). The combined action of replication and site-specific recombination between two copies of transposon is necessary to restore the original position of the first copy during the transposition which

<sup>1</sup> The real reason of nonlinear dependence of the level of cell resistance to Sm on the copy number of mini-Mu unit in *M. methylotrophus* chromosome is unclear. One of the possibilities could be a limitation of antibiotic transport facility, and so at the determined Sm<sup>R</sup> genes expression level, the corresponding protein products could degrade all Sm penetrated in the cell independently on extracellular Sm concentration.

seems less probable in the absence of the specific counter-selection against chromosomal rearrangements. Thus, in our experiments, we were unable to detect clones with the restored positions of the original copy of mini-Mu unit in the chromosome: both chromosomal DNA fragments carried mini-Mu unit and selected by Southern DNA hybridization in new strains differed in size from the corresponding fragment in the strain progenitor (Fig. 5). In case of restored position of the original mini-Mu unit, the new fragment had to be added to the previously detected one at the hybridization's pattern of the new strains. Therefore, all the clones tested in the present study underwent chromosomal rearrangements.

## Discussion

In the present study, the earlier constructed two-plasmid system for Mu-driven integration of recombinant DNA fragments in the *E. coli* chromosome (Akhverdyan et al. 2007) was adjusted for efficient exploitation in *M. methylotrophus*. Helper plasmids that are capable of autonomous replication in the methylotrophic cells and carry thermo-inducible genes for the Mu transposition factors are the first elements of the modified system. Introduction of these plasmids into *M. methylotrophus* can be performed by electroporation (data not shown) and conjugative transfer. The use of helpers based on replicons of different stability broadens the experimental potential of the system for genetic modification of strains. For example, the helper pAET7 which is stably maintained is suitable to use for multiple consecutive integrations of various mini-Mu units in the same strain or for the several cycles of amplification of an integrated copy of mini-Mu unit by the replicative transposition mechanism. At the same time, the use of the unstable helper, pTP310, is useful for one-cycle integration with rapid selection of a plasmidless recombinant strain.

The second element of the system is the integrative vectors that can be replicated in only *E. coli* strains. These can be introduced into *M. methylotrophus* by conjugative transfer or by electroporation and possess MCS in mini-Mu unit for cloning the genes of interest. The *kan* gene flanked with the *FRT* sites provides the selection of the mini-Mu unit integrants and can be excised if necessary from the final strain using pFLP31 plasmid bearing the FLP recombinase gene of *S. cerevisiae*.

The advantage of the system for consecutive integration of a few heterologous genes in the *M. methylotrophus* chromosome was demonstrated using the *amyE* gene from *B. amyloliquefaciens* and *xylE* gene from *P. putida* mt-2 as models. The amplification of the integrated genes in the *M. methylotrophus* genome using the mechanism of replicative transposition was also demonstrated, although the observed

efficiency of amplification was significantly lower than that under analogous conditions in *E. coli* (Akhverdyan et al. 2007). The following modification of the constructed integrative plasmid due to insertion of the small (about 400 bp) DNA fragment carrying phage Mu enhancer sequence (Yin et al. 2007) in the mini-Mu unit significantly increased efficiency of the possible intrachromosomal amplification: In the same conditions as described in the corresponding part of “Results”, we could detect the strains with two to six copies amplified mini-Mu units (Tokmakova et al., in preparation).

An adaptation of the Mu transposition system for use in the *M. methylotrophus* was based on the previously known functional homology of DNA-bending proteins from various bacteria and *E. coli* proteins HU and IHF (Swinger and Rice 2004) that are involved in formation of the transpososome structure along with phage-specific DNA elements (Mu-*attL/R*) and transposition factors (MuA and MuB; Gueguen et al. 2005). A priori, the simple insertion rather than the replicative transposition mechanism would operate most likely in *M. methylotrophus* because the former occurs via reparative instead of replicative DNA synthesis, and therefore, lower amounts of host-specific proteins are necessary. However, the predominant formation of cointegrate structures observed in the experiments on primary mini-Mu unit transposition and intrachromosomal mini-Mu unit amplification support the latter statement. In other words, it was experimentally shown that the *M. methylotrophus* proteins, (1) can function in the same way as their analogs in *E. coli* cells during transpososome formation and (2) ensure all the following stages of the mini-Mu unit replicative transposition. This makes it possible to employ Mu-driven integration and amplification of integrated recombinant DNA to practical applications.

Doubtless, the study of basic aspects of Mu-driven transposition in *M. methylotrophus* requires further experiments. However, the designed system is already being exploited in the modification of *M. methylotrophus* strains, and experiments on metabolic engineering (Tokmakova et al. 2007) focused on new biologically active and biotechnologically important substance production.

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