

Application of the bacteriophage Mu-driven system for the integration/amplification of target genes in the chromosomes of engineered Gram-negative bacteria—mini review

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Abstract The advantages of phage Mu transposition-based systems for the chromosomal editing of plasmid-less strains are reviewed. The *cis* and *trans* requirements for Mu phage-mediated transposition, which include the L/R ends of the Mu DNA, the transposition factors MuA and MuB, and the *cis/trans* functioning of the E element as an enhancer, are presented. Mini-Mu(LR)/(LER) units are Mu derivatives that lack most of the Mu genes but contain the L/R ends or a properly arranged E element *in cis* to the L/R ends. The dual-component system, which consists of an integrative plasmid with a mini-Mu and an easily eliminated helper plasmid encoding inducible transposition factors, is described in detail as a tool for the integration/amplification of recombinant DNAs. This chromosomal editing method is based on replicative transposition through the formation of a cointegrate that can be resolved in a recombination-dependent manner. (E-plus)- or (E-minus)-helpers that differ in the presence of the *trans*-acting E element are used to achieve the proper mini-Mu transposition intensity. The systems that have been developed for the construction of stably maintained mini-Mu multi-integrant strains of *Escherichia coli* and *Methylophilus methylotrophus* are described. A novel integration/amplification/fixation strategy is proposed for consecutive independent replicative transpositions of different mini-Mu(LER) units with “excisable” E elements in methylotrophic cells.

Keywords Chromosomal editing · Dual-component system · Enhancer element · Excisable marker · L/R ends · MuA transposase · Plasmid-less recombinant strain · Replicative transposition

Introduction

The relevance and practical significance of constructing plasmid-less recombinant bacterial strains for use in applied microbiology and biotechnology are increasing due to the potential for genetic instability to reduce the number of active recombinant alleles in plasmids (Friehs 2004) and restrictions on the application of plasmids in large-scale industries of the First World (European Council Directives 1990, 1998). *In vivo* chromosomal editing methods, which are primarily based on homologous and/or site-specific recombination of DNA as well as on transposition mechanisms, have been used to engineer plasmid-less bacteria (Balbás and Gosset 2001). The increase in genomic copy number of native or previously modified target genes is an important tool for chromosomal editing and the construction of stably maintained bacterial genomes. The development of new approaches for the integration (Minaeva et al. 2008; Rivero-Müller et al. 2007) and the increase in genomic copy number (Tyo et al. 2009) of recombinant DNA fragments remains relevant even for *Escherichia coli* and closely related Gram-negative bacteria for which recombineering-based technologies (Court et al. 2002; Sawitzke et al. 2007; Sharan et al. 2009) have already been developed to finely modulate the expression of chromosomal genes (De Mey et al. 2010; Katashkina et al. 2005; Meynial-Salles et al. 2005)

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and sometimes conditionally increase (Doroshenko et al. 2010b) or silence (Krylov et al. 2010) the level of their transcription. The methods of integration/amplification are especially important for bacteria for which the genetic tools for the chromosomal editing are not as comprehensive and diversified as those available for *E. coli*.

The integration of target genes in a bacterial chromosome followed by the increase in genomic copy number can be efficiently achieved using a phage Mu-driven transposition system originally developed for *E. coli* and initially characterized more than 20 years ago (Castilho et al. 1984; Chaconas et al. 1981a, b). Since the mid-1980s, several Mu derivatives, mini-Mu(s), have been constructed and extensively used for applications in classical in vivo transposition, including insertional mutagenesis, gene fusion, and mapping techniques, as well as for gene cloning and DNA sequencing strategies (Groisman 1991; Groisman and Casadaban 1986, 1987). The highly efficient Mu-based technology of the in vitro DNA transposition has been developed as a convenient tool for the functional analysis of genes, genomes, and proteins because of the accurate nature reaction and the low stringency for the target preference (Haapa et al. 1999; Haapa-Paananen et al. 2002; Savilahti et al. 1995; Savilahti and Mizuuchi 1996; Turakainen et al. 2009). The construction of recombinant plasmid-less L-threonine-overproducing *E. coli* strains may have been the first application of mini-Mu as a vector for the integration/amplification of target pathway genes for metabolic engineering (Kurahashi et al. 1990; Kurahashi and Takinami 1991).

Comprehensive investigations, primarily in vitro, of the Mu-driven system have provided extensive insights into the molecular aspects of the transposition mechanism. These works have been recently summarized in excellent research papers and overviews (Abdelhakim et al. 2008; Au et al. 2006; Chaconas and Harshey 2002; Choi and Harshey 2010; Gueguen et al. 2005; Harshey and Jayaram 2006; Nakai et al. 2001; Rice and Baker 2001). However, in vivo Mu-driven systems may be underutilized in applied microbiology and biotechnology. The main aim of this review is to highlight, especially for the new generation of investigators, the potential application of Mu-driven systems as a powerful genetic tool for the integration/amplification of target genes and the construction of plasmid-less engineered bacterial strains.

Cis and trans requirements of Mu phage-mediated transposition

Known as the most efficient transposon, Mu is a temperate bacteriophage that is capable of growth on many enteric bacteria, including *E. coli* K-12. The Mu phage undergoes two alternative transposition pathways (Fig. 1) at different

stages of its life cycle (Howe 1997; Paolozzi and Ghelardini 2006; Symonds et al. 1987). The Mu-driven “integrative” (or “conservative” or “nonreplicative”) transposition, which is also designated as a “simple insertion” (Akroyd and Symonds 1983; Harshey 1984; Liebart et al. 1982), and the “replicative” transposition (Chaconas et al. 1981b, 1996) are temporally separated. During the Mu phage infection step, various flanking sequences from the previous host are lost during the integrative transposition of the linear Mu DNA into a random site of the bacterial chromosome (Au et al. 2006; Bukhari and Zipser 1972). Replicative transposition through the formation of a “cointegrate” structure is obligatory for replication and for the production of approximately 100 phages during lytic growth (Chaconas et al. 1981b; Chaconas and Harshey 2002; Craigie and Mizuuchi 1985). As a result of replicative transposition, new copies of the Mu DNA occur at many sites in the bacterial genome, frequently within several kilobase pairs of one another. The most obvious factor that affects the random integration of Mu DNA into bacterial genes is their efficient transcription, which has a clearly negative impact on transposition (Manna et al. 2004).

Both Mu-mediated transposition pathways are catalyzed by a high-order protein–DNA complex called a transpososome (reviewed in Gueguen et al. 2005; Harshey and Jayaram 2006). The Mu DNA sites and proteins involved in transpososome assembly and function are presented in Figs. 2 and 3, respectively (for detailed references, see Chaconas and Harshey 2002; Harshey and Jayaram 2006). The core of the Mu transpososome is composed of two Mu end DNA segments (L and R ends) that are synapsed by stably bound multiple subunits of the transposase MuA, which catalyzes the specific DNA cleavage and joining required for transposition (Craigie et al. 1984; Kuo et al. 1991; Lavoie et al. 1991). The L/R ends each contain three MuA-binding sites (L1–L3 and R1–R3, correspondingly) with different spacings (Fig. 2a) (Craigie et al. 1984). MuA binds as a monomer to each L/R site, introducing an 80° to 90° bend (Kuo et al. 1991). The function of the *cis*- or *trans*-encoded MuA protein is essential for the transposition in vivo of the mini-Mu that is presented on supercoiled DNA and consists of the L/R sites (Patterson et al. 1986). The six binding sites are not equally important for transpososome assembly. Indeed, genetic experiments show the unimportance of the R3 site; a deletion of the L3 site results only in a 10-fold reduction in transposition in vivo (Groenen et al. 1985). It is shown in vitro that a MuA-mediated stable synaptic complex with only three (L1, R1, R2) sites could be formed in which the donor DNA strands are nicked and fully competent in the subsequent strand-transfer step of transposition (Kuo et al. 1991). The linear mini-Mu DNA flanked by the (R1R2) sites in an inverted orientation is efficiently used for in vitro MuA-mediated

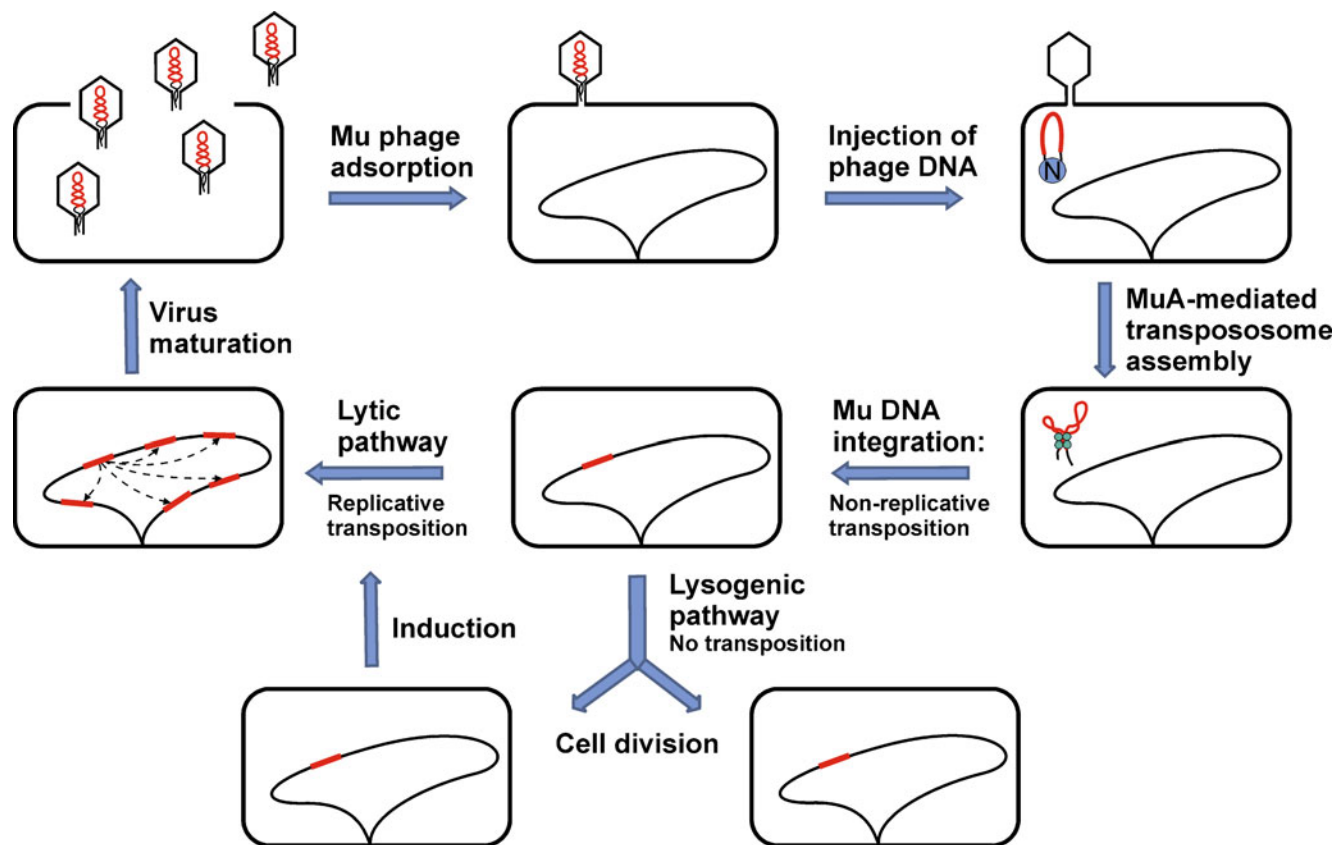


Fig. 1 The Mu phage life cycle. As a temperate phage, Mu can undergo lysogenic or lytic growth. The life cycle begins with infection in which the phage adsorbs and transfers its linear double-stranded DNA [37 kb Mu genome flanked by short regions from the previous host (Daniell et al. 1973; Bukhari and Taylor 1975)] to a bacterial cell. The ends of the infecting DNA are bound by the MuN protein to form a noncovalent circular structure (Gloor and Chaconas 1986; Harshey and Bukhari 1983). In an integrative transposition that requires the MuA protein, the flanking sequences are lost and the Mu DNA is

randomly inserted into the bacterial genome. After integration, approximately 1–10% of the phages become lysogens (Howe and Bade 1975). The majority of the phages immediately enter the lytic cycle (which is also the ultimate fate of the induced lysogens) during which MuA, assisted by the activities of several factors, catalyzes multiple replicative transpositions that eventually produce a burst size of approximately 100 new phages. Adapted from Choi and Harshey (2010) and Sokolsky and Baker (2003)

assemble the transpososome followed by integrative transposition in vitro (Haapa et al. 1999; Savilahti et al. 1995) or in vivo in genomes of different organisms (Lamberg et al. 2002; Paatero et al. 2008; Pajunen et al. 2005).

In *E. coli*, Mu transpososome assembly is facilitated by two host-encoded DNA bending proteins—HU and a sequence-specific integration host factor (IHF) (Swinger and Rice 2004). HU binds in vivo to the spacer between L1 and L2 (Lavoie et al. 1996), introducing a bend and presumably drawing the bound MuA protomers together (Gueguen et al. 2005). The binding site for IHF is in an E element that is located approximately 1 kb from the L end in the native Mu DNA.

The enhancer element, E [earlier named as the internal activation sequence or IAS (Mizuuchi et al. 1995; Mizuuchi and Mizuuchi 1989)], stimulates transposition more than 100-fold in vitro and in vivo (Castilho et al. 1984; Leung et al. 1989; Surette et al. 1989). The E element partially

overlaps with the O1–O3 region (Krause and Higgins 1986), which was initially identified as the operator to which the Mu c-repressor binds to silence the transcription of early phage functions from the P_e promoter (Fig. 2a). The E element is composed of two clusters of MuA-binding sequences separated by a binding site for IHF (Mizuuchi and Mizuuchi 1989; Surette et al. 1989). The site-specific IHF-mediated bending of DNA at the E element is presumed to assist MuA-mediated end-enhancer interactions (Harshey and Jayaram 2006).

IHF is required in vitro for the efficient transposition of the mini-Mu carrying the E element when the superhelical density (σ) of the donor DNA is low (Surette and Chaconas 1989), i.e., σ decreases from -0.05 (a character value for naked bacterial superhelical DNA) to a level of $\sigma \sim -0.025$ [this density is typical for the “restrained” protein-bound DNA molecule in vivo (Dillon and Dorman 2010; Pettijohn 1996; Pettijohn and Pfenninger 1980)].

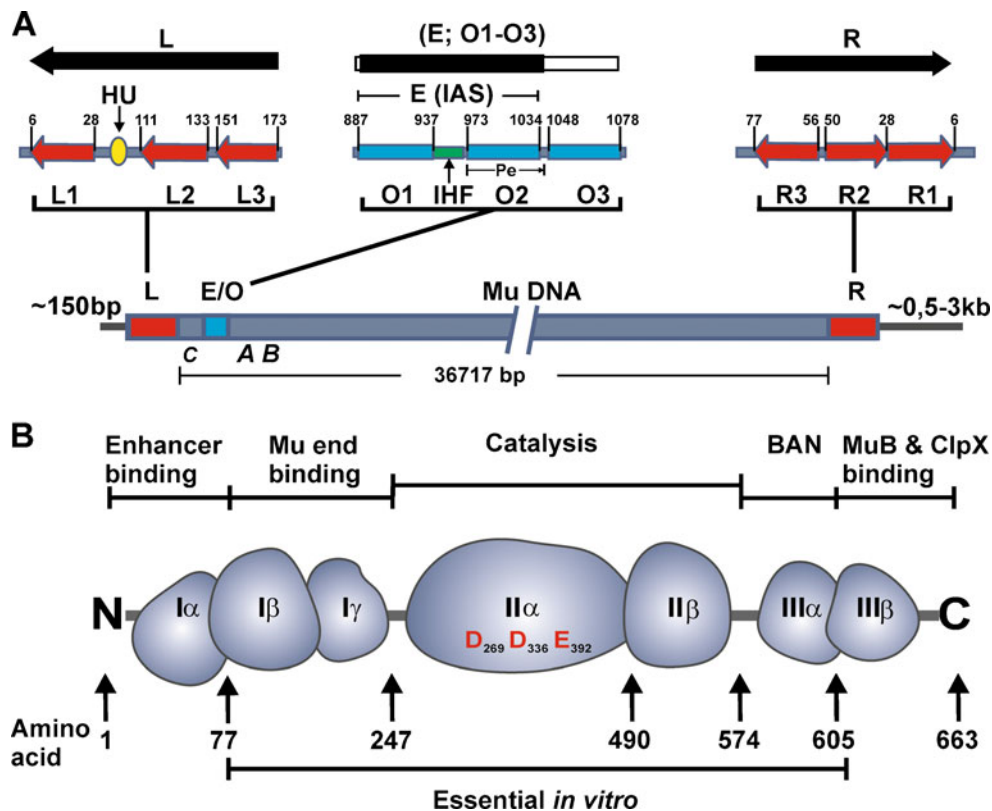


Fig. 2 *Cis* and *trans* requirements for Mu transposition. **a** Relative arrangement of subsites within the L/R ends and the E element on the 37-kb Mu genome (Morgan et al. 2002). The genes regulating transposition (*c*, *A*, *B*) and the *E. coli* proteins HU and IHF, which bind to the L end and the E element, respectively, during transposition, are indicated. The Mu transposase can interact with the high-affinity L/R sites and weak-affinity E sites. **b** The structural organization of the transposase MuA with the assigned functions of the various domains and subdomains. Based on limited proteolysis, three domains (I–III) have been assigned to MuA. Amino acid numbers corresponding to the boundary of each major domain are shown beneath the structure. The function of each domain is indicated at the

top. The N-terminal domain I contacts the L/R end and E element through separate regions. The central domain II of MuA is involved in catalysis (DDE in the catalytic triad). The C-terminal domain III is responsible for interaction with the MuB facilitating transposition factor and the host ClpX unfoldase protein. Isolated domain III displays, as well, the nonspecific DNA-binding and endonuclease activities designated as BAN (Wu and Chaconas 1995). The minimal region required for DNA binding, cleavage, and strand transfer *in vitro*, including subdomains I β , I γ , the catalytic core (domain II), and the N-terminal portion of domain III (III α), is indicated according to Krementsova et al. (1998). Adapted from Baker and Luo (1994), Choi and Harshey (2010), and Haapa-Paananen (2002)

The *in cis* orientation of the E element in mini-Mu with respect to the L/R ends is critical to its function, while its distance from the ends does not seem to be significant. The E element can stimulate transposition if it is present *in trans* in an unlinked DNA molecule (Surette and Chaconas 1992). The presence of the E element is important for correct transpososome assembly (Fig. 3) (Allison and Chaconas 1992; Lavoie and Chaconas 1995; Watson and Chaconas 1996), and it remains associated with this complex throughout the transposition process (Pathania et al. 2002, 2003; Yin et al. 2007).

MuA is a 663-aa residue protein that can be divided into three domains by partial proteolysis; each domain can be functionally or structurally divided into subdomains (Fig. 2b, and reviewed in Rice and Baker 2001). The N-terminal domain of the MuA protein contacts the sequences of the L/R ends and the E element through separate regions

(Mizuuchi et al. 1995). The central domain is involved in the catalysis of the transposition reactions. The C-terminal domain is responsible for the interaction with the host protein, ClpX (see below), and with the auxiliary transposition factor, the MuB protein. MuB not only modulates the activity of MuA (Faalen et al. 1978) but also delivers the target DNA to the transpososome (Chaconas and Harshey 2002; Roldan and Baker 2001) and protects the actively replicating/transposing Mu from self-integration (Ge et al. 2010; Han and Mizuuchi 2010). Both the DNA binding and activation of the MuA transposase functions of MuB are required for productive phage Mu replicative transposition, but only the activation of transposase is necessary for efficient integrative transposition (Roldan and Baker 2001). The MuB protein is not required for the integrative transposition (O'Day et al. 1978); however, it enhances the rate and extent of this pathway (Roldan and Baker 2001).

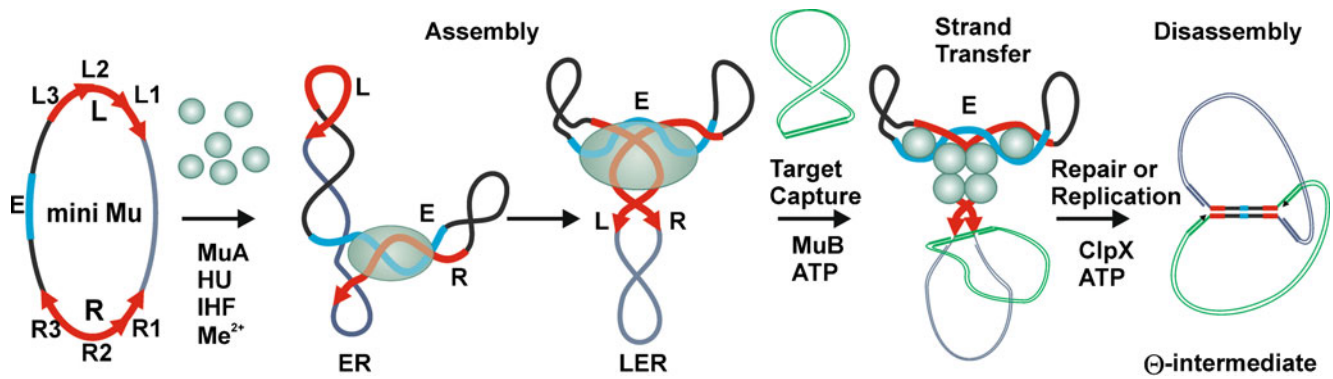


Fig. 3 The simplified scheme of the Mu-driven transposition pathway. The assembly of the Mu transpososome is initiated by the interaction of the MuA subunits with the R end and the E element. HU promotes capture of the L end by MuA/ER complex to form the MuA/ LER complex. MuA then acts *in trans* to first cleave and then join the cleaved ends of the Mu DNA to the target DNA. MuB captures and delivers the target DNA to the transpososome where the newly created

3'-OH groups now function in the joining or strand-transfer step catalyzed by the complex and ClpX-mediated exchanges MuA with a set of host proteins in preparation for repair or replication to finalize the integrative or replicative transposition pathways, respectively. For details, see Au et al. (2006), Choi and Harshey (2010), and Harshey and Jayaram (2006)

During the multistep reaction, the MuA present in the transpososome complex catalyzes the specific DNA single-stranded cleavages at the terminal CA dinucleotides of the L1 and R1 sites, using water as the nucleophile in such a way that 3'-OH groups of the transposable element are exposed (Mizuuchi 1984). These 3' ends serve as nucleophiles for the subsequent joining or strand-transfer step, attacking phosphodiester bonds spaced 5 bp apart on target DNA in a one-step transesterification reaction (Mizuuchi and Adzuma 1991). The resulting θ -like DNA structure is a common intermediate for both transposition pathways (Chaconas and Harshey 2002; Harshey and Jayaram 2006). The host ClpX unfoldase ultimately destabilizes the transpososome, which facilitates the recruitment of the host-dependent DNA replication/repair machinery to finalize the “nick–join–replicative” or “nick–join–repair” transposition pathways (Fig. 4) (Abdelhakim et al. 2008; Nakai et al. 2001). In both cases, the strand transfer at staggered positions in target DNA strands will cause a duplication of 5 bp of the target DNA flanking the transposed Mu-based element. Probably, ClpX participates, as well, in stimulating the nuclease activity of the C-terminal domain of the transposase MuA (Wu and Chaconas 1995) in a highly regulated reaction that removes the attached host DNA after the incoming Mu genome has inserted into a bacterial chromosome according to the integrative transposition (Choi and Harshey 2010).

Mini-Mu as a genetic tool for *E. coli* chromosomal editing

The structures of mini-Mus can differ significantly. Some mini-Mus carry all of the genetic elements essential for transposition, replication, and packaging. Other derivatives

possess only the Mu ends (later designated as mini-Mu(LR) units) and can be complemented in *trans* to perform Mu-specific functions in *E. coli* (Chaconas et al. 1981a; Harshey 1983; Patterson et al. 1986). Mini-Mu(cts) chromosomal vectors, which lack the genes required for

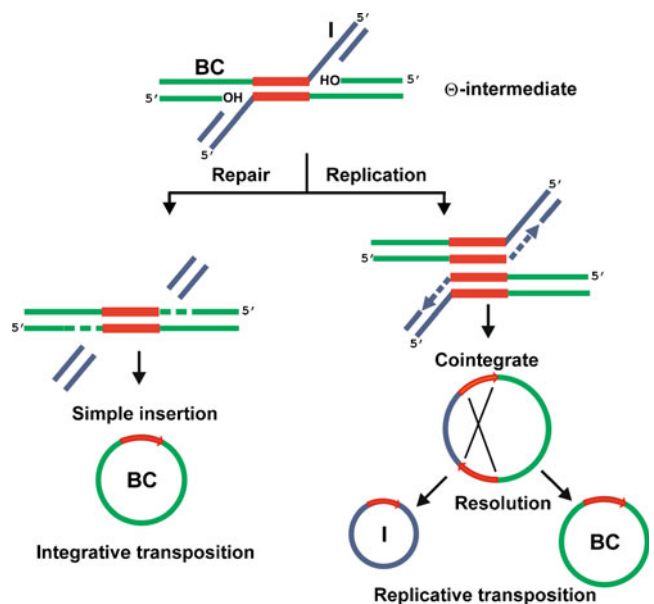


Fig. 4 The two outcomes of Mu-driven transposition. The common θ intermediate can be resolved differently by the DNA repair/replication host-dependent machinery through integrative or replicative transposition. Transposition of the mini-Mu(LR) unit from the integrative plasmid (*I*) into the bacterial chromosome (*BC*) results in a “simple insertion” in which *BC* gains a copy of the unit 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. The cointegrate can subsequently be resolved by homologous recombination between two mini-Mu units. Adapted from Abalagina et al. (2008a) and Choi and Harshey (2010)

making phage particles, have been used for simultaneous thermo-induced replicative transposition and heterologous protein synthesis in *E. coli* (Weinberg et al. 1993).

However, the *cis*-encoded transposition factors in the mini-Mu(*cts*) constructs can cause instability in engineered strains even under noninduced conditions (Akhverdyan et al. 2007). Stabilization has been achieved through the use of dual-component systems in which the genes for the MuA and MuB transposition factors are encoded *in trans* on an unlinked/nontransposed DNA molecule and eliminated after mini-Mu transposition.

Different variants of this system have been developed (Akhverdyan et al. 2007; Castilho et al. 1984; Chaconas et al. 1981a; Groenen et al. 1985; Patterson et al. 1986). One of the most popular systems includes an “integrative” plasmid that consists of a mini-Mu(LR) unit as the first component and a compatible “helper” plasmid carrying the inducible MuA and MuB genes as the second component. Usually, the helper plasmid possesses an unstable replicon and can be easily eliminated from the cells. The transposed genes in a set of mini-Mu(LR) units are flanked by Rho-independent transcription terminators in a directly repeated orientation (Abalakina et al. 2008a, b; Gulevich et al. 2009). The upstream terminator prevents readthrough transcription of the randomly integrated mini-Mu unit from the chromosomal promoter. In turn, the downstream terminator interrupts internal readthrough transcription of the mini-Mu that could interfere with transpososome assembly *in vivo* (Patterson et al. 1986).

In the mini-Mu derivatives that could be assigned as mini-Mu(LER) units, an E element properly arranged between the L and R ends positively influences transposition (Leung et al. 1989). However, intense expression of the MuA and MuB genes, even when located *in trans*, leads to host cell lethality because of the overly efficient replicative transposition of the mini-Mu(LER) units (Akhverdyan et al. 2007; Lee 2002). Although artificial mini-Mu(LER) units are actively used in fundamental investigations of transpososome assembly and function *in vitro* and *in vivo* (Lee 2002), they have not been widely applied in stably engineered *E. coli* strains.

In contrast, *in trans* E element has been successfully used to significantly increase the transposition efficiency of the mini-Mu(LR) unit. A comparison of the E-carrier ((E-plus)-helper¹) and the helper that did not contain the E element ((E-minus)-helper²) revealed a nearly two-order

magnitude of increase in the transposition efficiency of the mini-Mu(LR) units when the (E-plus)-helper was used.

To facilitate the selection of the transposed mini-Mu, an antibiotic resistance marker (Ant^R) can be included.³ Markers that are flanked by the sequences essential for site-specific recombination, $\lambda attL/R$ (Peredelchuk and Bennett 1997), *loxP* (Arakawa et al. 2001), or *FRT* (Datsenko and Wanner 2000), are usually used. These markers can be excised *in vivo* from the mini-Mu units by the corresponding recombinase to obtain the marker-less recombinant strain (Abalakina et al. 2008a; Gulevich et al. 2009; Wei et al. 2010).

Several protocols have been developed for mini-Mu(LR) unit transposition into the *E. coli* chromosome with the help of the dual-component Mu-driven system. The best results have been obtained when an (E-plus)-helper plasmid is first transferred into the recipient strain and is stably maintained under noninducing conditions. Then, an integrative plasmid is transferred into the helper-carrier strain; the efficacy of this step mainly determines the total efficiency of the detected mini-Mu(LR) unit transposition. Therefore, different strategies that are based on the presence of a selective marker in the mini-Mu(LR) unit are employed. For a marker-carrier mini-Mu, replication of the integrative plasmid is not necessary in the recipient cell. So, the transposase can be induced during plasmid entry followed by direct selection of the clones-integrants. Usually, transposition occurs in the majority of cells that receive the integrative plasmid (Zimenkov et al. 2004).

If the marker-less mini-Mu(LR) unit is used, the integrative plasmid has to be selectively transformed into the helper-carrier recipient. The transposase is then expressed in the cells that contain both autonomously replicating components of the Mu-driven system. Finally, the obtained clones-integrants are cured of the unstable plasmid(s). Usually, the transposition of the mini-Mu(LR) units can be detected in at least 10% of the induced cells (Savrasova et al. 2007).

Both strategies can lead to high-efficiency formation of clones possessing one or more (up to five to ten) copies of the mini-Mu(LR) unit in the chromosome (Akhverdyan et al. 2007). The copy number of the integrated mini-Mu can be increased further by reintroducing and expressing the (E-plus)-helper plasmid in the corresponding plasmid-less strain-integrand. The (E-minus)-helper plasmid has a significantly decreased but still detectable ability to facilitate intrachromosomal replicative transposition of mini-Mu(LR) units in *E. coli* (Gak et al., in preparation).

¹ The p15A-based plasmid pMH10 was used as a helper in this dual-component system (Akhverdyan et al. 2007). pMH10 carried the fragment from pMud4041 (Symonds et al. 1987), which consisted of the Muets62 repressor gene and MuA, MuB with the native *cts*-controlled regulatory region including P_c promoter, and (E; O1–O3).

² MuA and MuB genes controlled by the $\lambda P_L/O_L$ regulatory region were located in the nontransposing part of the single plasmid that carried the transposed mini-Mu(LR) unit as well (Patterson et al. 1986).

³ However, the efficiency of the developed dual-component Mu-driven system in *E. coli* is frequently so high that integrants of interest can easily be selected by total screening without using special Ant^R markers in the mini-Mu unit (Akhverdyan et al. 2007; Savrasova et al. 2007).

A dual-component Mu-driven system has been frequently used for *E. coli* chromosome editing and for constructing different bacterial strains for basic research and metabolically engineered amino acid production (Table 1). In most cases, the expression level of the Mu-integrated genes increased proportionally as the copy number in the chromosome increased. As a rule, the amplification of a biosynthetic operon leads to the increased accumulation of the corresponding amino acid. The amino acid production levels of the plasmid-less strains obtained were comparable to those of their

plasmid-carrier recombinant analogs, and the former were significantly more stable during nonselective cultivation (Akhverdyan et al. 2007; Savrasova et al. 2007).

The transposition of the mini-Mu(LR) unit into the bacterial chromosome mainly occurs through a “nick–join–replicative” mechanism with the formation of a cointegrate in which the integrative plasmid and the chromosome fuse and two copies of the mini-Mu unit border the junction of the fused replicons as direct repeats (Fig. 4) (Abalakina et al. 2008a; Patterson et al. 1986; Tokmakova 2010). The cointegrate is subsequently resolved by a reciprocal

Table 1 The list of several native and artificial genes/operons inserted into bacterial genomes using the dual-component Mu-driven integration/amplification system

Integrated gene(s)	Copy number	Purpose of the recombinant strain	Reference	
Integration into <i>E. coli</i> genome	$P_{lac} \rightarrow lacI$	1	Investigation of an artificial autoregulated system	Skorokhodova et al. 2004
	$P_{lac-lacI-ilvA}^{7434}$	1	Investigation of L-threonine catabolism	Sycheva et al. 2003
	Km ^R carrier mini-Mu(LR) units	1	Investigation of integration points determination	Zimenkov et al. 2004
	$thrA^*BC-Cm^R$	1–10	Basic research	Akhverdyan et al. 2007
	$P_R-thrA^*BC-Cm^R$	1–10	L-threonine production	ibid.
	$P_R-leuA^*BCD-Cm^R$	1–10	L-leucine production	ibid.
	<i>yddG</i>	1	Investigation of aromatic amino acid export	Doroshenko et al. 2007
	$P_{nlpD-yeaS}$	≥1	Investigation of branched-chain amino acids export	Kutukova et al. 2005
	P_R-leuA^*BCD	1–5	Amino acid production	Savrasova et al. 2007
	$P_R-pgi-pfkA$	≥1	Metabolic engineering	ibid.
	P_R-glK	≥1	Metabolic engineering	ibid.
	P_R-eno	≥1	Metabolic engineering	ibid.
	$P_R-ppc(ppc^*)$	≥1	Metabolic engineering	ibid.
	$P_{tac-aroG4-serA5}$	1	L-tryptophan production	Gulevich et al. 2009
	$P_R-ilvGMED$	4	Amino acid production; recipient for basic research	Eremina et al. 2010; Savrasova et al. 2006
	$P_R-prs (prs^*)$	≥1	L-histidine production	Klyachko et al. 2008
	$aroG4-pheA^B-aroL$	1	L-phenylalanine production	Doroshenko et al. 2010a, b
	$P_R-ilvGMED$	1	Isobutanol production via the L-valine biosynthetic pathway	Savrasova et al. 2011
	<i>scrKYABR</i>	≥1	To improve the sucrose uptake	Livshits et al. 2005
	$P_R-ilvGMEDA*YC$	≥1	L-isoleucine production	Savrasova et al. 2007
Integration into <i>M. methylotrophus</i> genome	$FRT-Km^R-FRT$	1	Basic research	Abalakina et al. 2008a
	Km ^R - <i>xylE</i> _{<i>P. putida</i>}	1	Basic research	ibid.
	<i>amy</i> _{<i>B. amyloliquefaciens</i>} -Km ^R	1	Basic research	ibid.
	<i>amy</i> + <i>xylE</i>	1+1	Basic research	ibid.
	Sm ^R (in mini-Mu(LR) units)	1–2	Basic research	ibid.
	<i>aroP</i> _{<i>E. coli</i>}	1	Enhancement of aromatic amino acid transport into the cell	Yomantas et al. 2010; Tokmakova et al. 2010
	ZsGreen _{<i>Zoanthus sp.</i>} , Sm ^R (in mini-Mu(LR) units)	1–2	Basic research	Tokmakova 2010
	ZsGreen, Sm ^R (in mini-Mu(LER) units)	1–6	Basic research	ibid.

recombination event between the two mini-Mu units. The chromosomal structure of the final strain that contains one copy of the mini-Mu unit is the same as if integrative transposition of this unit had occurred. The sites of mini-Mu insertion in the *E. coli* chromosome could be precisely determined (Wei et al. 2010; Zimenkov et al. 2004) by inverse polymerase chain reaction (Ochman et al. 1988).

Several copies of the mini-Mu(LR) units can be easily detected in the bacterial genome after using the dual-component Mu-driven system. It could not be excluded that these multiple copies are due to independent transpositions from several copies of the nonreplicated integrative plasmid that were initially transformed into one cell or from several copies of autonomously replicated integrative plasmid. However, especially when the nonreplicating integrative plasmid was used, it seems more likely that the amplification is achieved due to the mini-Mu intrachromosomal replicative transposition. Certainly, the genome of the final stable plasmid-less multi-integrant strain may contain transposition-mediated chromosomal rearrangements, including inversions and/or deletions of bacterial DNA fragments (Watson et al. 2004).

Adaptation of the Mu-driven system for *Methylophilus AS1*

Interest in the use of methylotrophic bacteria for applied microbiology and biotechnology has increased in recent years (Schrader et al. 2009). Several genomes of methylotrophs have been sequenced (Chistoserdova et al. 2007; Vuilleumier et al. 2009). Significant progress has been made in elucidating the metabolism of these bacteria (Chistoserdova et al. 2009), and the number of tools available for genetic and metabolic engineering has expanded greatly (Bélanger et al. 2004; Choi et al. 2006; Marx and Lidstrom 2001, 2004). Strategies to produce fine and bulk chemicals using methylotrophs have been described previously (Bourque et al. 1995; Fitzgerald and Lidstrom 2003; Motoyama et al. 1993, 2001). In particular, the obligate methylotroph *Methylophilus methylotrophus* AS1, which was extensively studied with respect to the industrial-scale production of single cell protein from methanol in the 1970s (Anthony 1982; Vasey and Powell 1984), was recently metabolically engineered for the biosynthesis of L-lysine (Gunji and Yasueda 2006; Tsujimoto et al. 2006) and L-phenylalanine (Tokmakova et al. 2010) and for the efficient secretion of recombinant proteins (Itaya et al. 2008).

The adaptation of mini-Mu transposition for chromosomal editing in *M. methylotrophus* AS1 (Abalakina et al. 2008a, b) was a significant development. Mu-driven integration of the *E. coli* transporter gene into the

methylotrophic genome was the basis for the development of an efficient method of constructing auxotrophic mutants (Yomantas et al. 2010) with the following exploiting the developed system for metabolic engineering of L-phenylalanine overproduction (Iomantas and Abalakina 2002; Tokmakova et al. 2008, 2010).

The adaptation of the Mu-driven system to *M. methylotrophus* AS1 included several stages (Abalakina et al. 2008a). Initially, a DNA fragment containing the genes from *E. coli* plasmid, pMH10 (Akhverdyan et al. 2007), for the thermo-induced transposition factors was cloned into vectors containing the broad host range replicons of the IncQ (Chistoserdov and Tsygankov 1986) or IncP α groups (Ditta et al. 1985; Pansegrau et al. 1994). These new (E-plus)-helpers (Fig. 5a) were transformed into methylotrophic cells (by mobilization or electroporation) and maintained under strictly selective conditions without expressing the Mu transposition factors. All of the helpers could be easily eliminated from *M. methylotrophus* AS1 by aerobically culturing the cells in liquid medium without antibiotics (Abalakina et al. 2008a).

The constructed integrative plasmids could not autonomously replicate in methylotrophic cells. Because it was unclear if it was possible for Mu-driven transposition to occur, the Mob_{RP4}⁺ element (Simon et al. 1983, 1984) was included in the initial integrative plasmids to ensure their highly efficient transfer into *M. methylotrophus* by mobilization (Abalakina et al. 2008a). The next generation of integrative plasmids (Fig. 5b, see experimental details in Tokmakova 2010) did not carry the Mob⁺ element and could be autonomously maintained only in the *pir*⁺ *E. coli* strain used to propagate γ -replicon originating from R6K (Bowers et al. 2007). These integrative plasmids were introduced into the methylotrophic recipient cells by electroporation. The Km^R gene, flanked by *FRT* sites, was used as the “excisable” selective marker in the constructed mini-Mu(LR) units (Abalakina et al. 2008a).

The mini-Mu(LR) unit can be detected in the *M. methylotrophus* AS1 chromosome after the transfer of the integrative plasmid into the helper-carrier cells in the presence of the partially induced (37°C) transposition factors. Curing the selected integrants of the helper plasmid and Flp-mediated elimination of the marker results in a plasmid-less marker-less recombinant strain that is ready for the next round of mini-Mu transposition (Abalakina et al. 2008a).

The efficiencies (10^{-2} – 10^{-3}) of plasmid mobilization and Mu-driven integration are correlated. This means that, under the developed conditions, a mini-Mu(LR) unit is transposed into the bacterial chromosome of each cell of the recipient strain that received the integrative plasmid. As in *E. coli*, the transposition of the mini-Mu(LR) unit into the *M. methylotrophus* AS1 chromosome occurs through

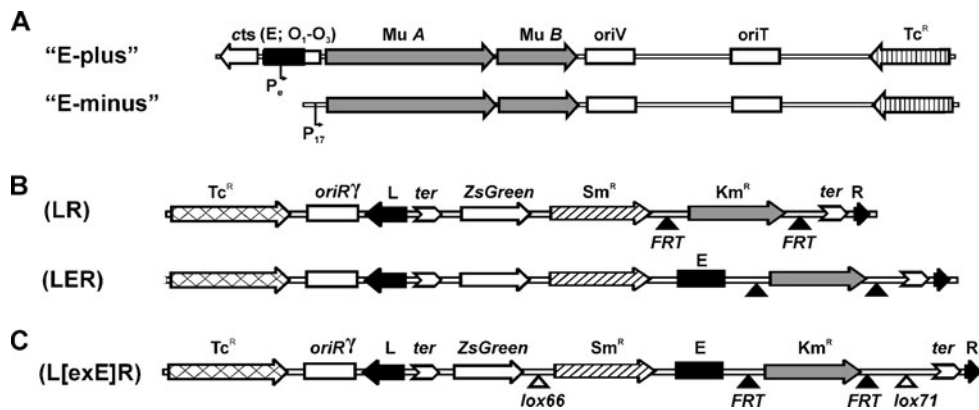


Fig. 5 Schematic linear representation of the set of helper (a) and integrative (b, c) plasmids used in the dual-component Mu-driven system for integration/amplification of genes in the *M. methylotrophus* AS1 genome. These mini-Mu units carry excisable Km^R marker bracketed by *FRT* sites, Sm^R marker used for selective amplification, and a gene of *ZsGreen* fluorescent protein (Matz et al. 1999) as a reporter. The internal parts of all mini-Mu units are bracketed by Rho-independent transcription terminators (*ter*). **a** The helpers are based on the mobilizable (*oriT*) replicon (*oriV*) of the IncP α group plasmid pRK310 (Ditta et al. 1985). The (E-plus)-helper, pTP310 (Abalakina

et al. 2008b), carries the *Mu*cts62 and *MuA* and *MuB* genes under the native Mu DNA early regulatory region, including the P_e promoter and (E; O1-O3) (Krause and Higgins 1986). In the (E-minus)-helper, p17TP310, *MuA* and *MuB* are under the transcriptional control of the *M. methylotrophus* constitutive P_{17} promoter (Abalakina et al. 2008a). **b** The integrative plasmids containing the mini-Mu(LR) or (LER) units are based on the conditionally replicated *pir*⁺-dependent (*oriR* γ) plasmid pAH162 (Haldimann and Wanner 2001). **c** One of the new integrative plasmids with a mini-Mu(L[exE]R) unit in which the E element is flanked by *lox66/71* sites

“nick–join–replicative” formation of the cointegrate, followed by its recombination-mediated resolution (Abalakina et al. 2008a). These factors suggested that the mini-Mu(LR) units might be amplified in *M. methylotrophus* with the assistance of the same set of host proteins (Au et al. 2006; Gueguen et al. 2005; North and Nakai 2005). However, the *MuA*- and *MuB*-mediated intrachromosomal duplication of the mini-Mu(LR) unit was detected only with a low (10^{-4}) frequency (Abalakina et al. 2008a).

The efficiency of the Mu-driven increase in genomic copy number in *M. methylotrophus* AS1 was increased 100-fold by exploiting the mini-Mu(LER) unit (Fig. 5b). When transferred as part of the integrative plasmid into *M. methylotrophus*, these units, like earlier used mini-Mu (LR), could be integrated into the bacterial chromosome with the formation of a cointegrate; the transposition efficiency of this process was equally high whether the helper was (E-plus) or (E-minus). On the contrary, the increase in genomic copy number efficacy of the mini-Mu (LER) or mini-Mu(LR) units was significantly dependent on the type of helper used (Table 2). The detected capacities for increase in genomic copy number for the mini-Mu (LER) and mini-Mu(LR) units differed by approximately four orders of magnitude in the presence of the expressed (E-minus)-helper.

These results served as the basis for a novel integration/amplification/fixation strategy that was developed for the *M. methylotrophus* AS1 Mu-driven system. The E element in the mini-Mu(LER) unit was substituted with its excisable analog, which was bracketed by *loxP*-like sites [*lox66* and *lox71* (Albert et al. 1995)] that could serve as the target for

irreversible excision by phage P1 Cre recombinase (Abremski and Hoess 1984). Thus, the mini-Mu(L[exE]R) unit (Fig. 5c) could be integrated and amplified in the *M. methylotrophus* AS1 genome according to the standard procedure (using (E-plus)- or (E-minus)-helpers), followed by Cre-mediated excision of the E element. The “residual” copies of the mini-Mu(LR) units in the chromosome could not be further efficiently amplified in the presence of the (E-minus)-helper plasmid. This allows the integration and independent amplification of another mini-Mu(L[exE]R) unit into the *M. methylotrophus* AS1 genome without changing the number and location of the previously integrated genes (Fig. 6) (Tokmakova 2010).

There are still many fundamental questions concerning the differences in the efficiencies of the Mu-driven increase in genomic copy number in *E. coli* and *M. methylotrophus*. The answers likely depend on the precise nature of the respective transpososome assembly processes. These pro-

Table 2 Efficiency of the mini-Mu units amplification in the *M. methylotrophus* AS1 chromosome in dependence on the helpers’ structure

Mini-Mu unit \ Helper	(E-plus)	(E-minus)
(LR)	$\sim 10^{-4}$	$\sim 10^{-6}$
(LER)	$\sim 10^{-2}$	$\sim 10^{-2}$

The increase in genomic copy number efficiency was evaluated as the part of multi-integrants obtained after induced transposition factors expression in the single-integrant cells selected on the media with increased concentrations of streptomycin (2 mg/ml of Sm) used as a selective marker according to Abalakina et al. (2008a)

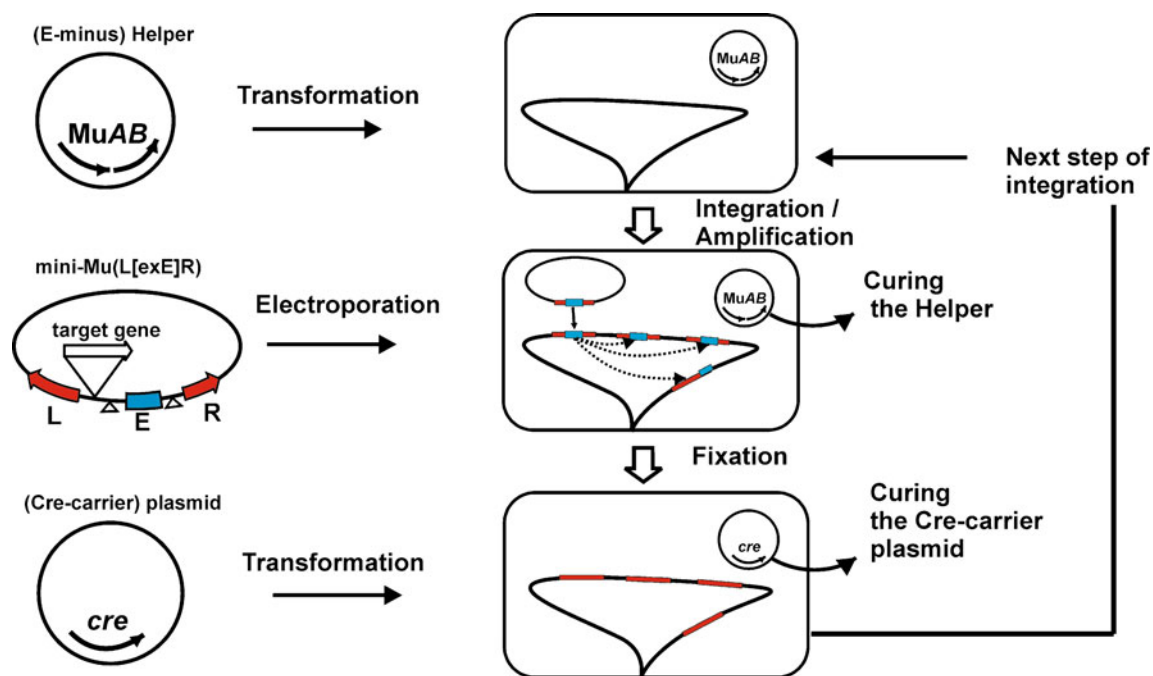


Fig. 6 Application of the integration/amplification/fixation strategy. Initially, a mini-Mu(L_{ex}E]R) unit is integrated and amplified in the chromosome of *M. methylotrophus* AS1 according to the replicative transposition in the presence of the expressed (E-minus)-helper plasmid. Then, the E element is excised by Cre-mediated site-specific recombina-

tion. The “truncated” mini-Mu(LR) units could be further amplified with very low frequency by (E-minus)-helper, i.e., their positions in the chromosome could be considered as fixated. So, the obtained multi-integrant could be served as the recipient for integration and independent amplification of new Mu(L_{ex}E]R) units

cesses could depend, in particular, on the intracellular concentrations of the expressed transposition factors and the DNA-bending host proteins that facilitate transposition at the “restrained” host DNAs.

Concluding remarks

We have reviewed the adaptation of the dual-component Mu-driven system for chromosomal editing and the construction of plasmid-less marker-less strains of several Gram-negative bacteria due to the integration/amplification of target genes by the “nick–join–replicative” pathway in vivo. Certainly, other methods could be used for the chromosomal amplification of the target genes as well. The same purposes could be achieved through the single insertion of a DNA cassette with multiple copies of the target gene (Choi et al. 2006). The marker-containing single insertions in different specific (Haldimann and Wanner 2001; Minaeva et al. 2008) or random (De Lorenzo and Timmis 1994; Peredelchuk and Bennett 1997; Wei et al. 2010) sites could be combined in one strain by general P1-mediated transduction, followed by marker curing. These alternative methods, each of which possesses its own specific advantages, are significantly inferior to the Mu-driven amplification approach with respect to ease-of-use and the speed with which a target can be achieved,

especially if selection for the best variants of the multi-integrant is possible.

Up today, in vitro-assembled Mu transpososome complexes have been efficiently applied for the in vivo random insertion of recombinant DNAs into different bacterial genomes by the “nick–join–repair” pathway (Haapa et al. 1999; Laasik et al. 2005; Lamberg et al. 2002; Lanckriet et al. 2009; Pajunen et al. 2005; Savilahti et al. 1995; Savilahti and Mizuuchi 1996; Tu Quoc et al. 2007; Wei et al. 2010; Wu et al. 2009). Moreover, efficient Mu transpososome-based integration has been verified even in yeast, mouse, and human genomes (Paatero et al. 2008; Turakainen et al. 2009). Many more host proteins likely participate in the process of Mu-driven replicative transposition than in the simple insertion of mini-Mu into the host chromosome (Au et al. 2006; North and Nakai 2005). It is possible that only integrative, but not replicative, transposition occurs in this broad range of host organisms, even when efficient intracellular expression of Mu transposition factors is provided. However, to broaden the range of hosts in which the Mu-driven system can be used for the integration/amplification of target genes, its adaptation should be attempted, especially by using mini-Mu(LR)/(LER) units in combination with different (E-plus)/(E-minus)-helpers expressed in different genetic backgrounds.

The combined Mu-driven system could be proposed when the in vitro-assembled MuA-mediated transpososome

results single-copy integrants due to the integrative transposition, followed by the increase in genomic copy number of the integrated mini-Mu in vivo by the replicative transposition in the presence of the helper plasmid expressing MuA and MuB factors.

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