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In vivo DNA cloning and adjacent gene fusing with a mini-Mu-*lac* bacteriophage containing a plasmid replicon

(transcription-translation/gene regulation/transposition/β-galactosidase/*Escherichia coli*)

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ABSTRACT **A mini-Mu bacteriophage containing a high copy number plasmid replicon was constructed to clone genes *in vivo*. A chloramphenicol resistance gene for independent selection and the *lacZYA* operon to form gene fusions were also incorporated into this phage. This mini-Mu element can transpose at a high frequency when derepressed, and it can be complemented by a helper Mu prophage for lytic growth. DNA sequences that are flanked by two copies of this mini-Mu can be packaged along with them. After infection, homologous recombination can occur between the mini-Mu sequences, resulting in the formation of plasmids carrying the transduced sequences. *lac* operon fusions can be formed with promoters and translation initiation sites on the cloned sequences in the resulting plasmids. The utility of this system was demonstrated by cloning genes from eight different *Escherichia coli* operons and by identifying *lac* fusions to the regulated *araBAD* operon among clones selected for the nearby *leu* operon.**

The cloning of DNA sequences has become an important step in many biological studies. This has usually involved the isolation of appropriate DNA fragments and their joining to vector DNA sequences capable of replication when introduced into an appropriate host. Transposable elements have been used to translocate and clone genes *in vivo* by a process involving multiple transposition steps that results in two copies of the transposon flanking the cloned DNA segment. This structure was usually placed on a new replicative element such as a conjugative plasmid because transposable elements do not usually have replicons (1–3). To eliminate the need for such a complex and potentially unstable structure, we have incorporated a plasmid replicon inside the transposable element bacteriophage Mu.

Bacteriophage Mu is a temperate phage that undergoes transposition hundreds of times when it replicates (4–6). Mu DNA is packaged by a head-full mechanism that starts at the Mu left end and incorporates phage and adjacent host DNA up to a total of 39 kilobase pairs (kb). Mu genomes that have deletions are encapsidated with correspondingly more adjacent DNA. We have used these properties of bacteriophage Mu to develop an *in vivo* cloning system that also incorporates the bacteriophage Mu-*lac* gene fusion technology (7–9). This modified bacteriophage allows the formation of clones that are analogous to the ones constructed *in vitro*, where the DNA sequence is simply joined to a replicating vector. The *lac* gene fusions formed can be used to study gene structure and expression (10, 11), and the resulting hybrid proteins with β-galactosidase activity are useful for studying properties of the original protein (12, 13). Here, we report the cloning of several *Escherichia coli* genes and the formation of a regulated *lac* fusion to a particular gene.

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Table 1. Bacterial strains, bacteriophages, and plasmids

	Description	Ref. or source
Bacterial strains*		
DH37	F⁻ Δ(*his attP2H*)*hsdR trp met*	14
M8820	F⁻ *araD139* Δ(*araCOIBA leu*)7697 Δ(*proAB argF lacIPOZYA*)XIII *strA*	15
M8834m	F⁻ Δ(*araCO leuABCD*)1109 *strA mal*(*T* or *K*)::Mucts	15
MC4100	F⁻ *araD139* Δ(*argF lacIPOZYA*)U169 *strA relA fla*	16
MC4100-3G	NC4100 *arg*::Mucts	Lysogeny
XPh43	F⁻ Δ(*argF lacIPOZYA*)U169 *trp* Δ(*brnQ phoA proC phoB phoR*)24	17
Phages†		
Mucts	Mu with temperature-sensitive repressor *c*ts62	18
MudII1681	Mucts62 A^+ B^+ Km^r *lac*('*ZYA*)931	8
MudII4042	Mucts62 A^+ B^+ Cm^r *rep*P15A *lac*('*ZYA*)931	pBC4042 Fig. 1
Plasmids		
pACYC184	Cm^r Tc^r *rep*P15A	19
pBCO	pUC9 with *Hae* II–*lac* fragment removed	This work
pBC1	pBCO::MudII1681	8
pBC4042	pBCO::MudII4042	Fig. 1
pEG109	MudII4042::*phoA proC*	This work
pEG176	MudII4042::*arg*	This work
pUC9	Ap^r *lac*'*IPOZ*'*rep*pMB1	20

Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; ^{r}, resistant; A^+ B^+, Mu transposition-replication genes.
E. coli gene designations are summarized by Bachman (21). In the text, Mucts following a strain name indicates a lysogen. *ara* alleles are from *E. coli* B. Mutants of *argF* are not arginine-requiring because *E. coli* K-12 has an isozyme gene *argI*.
†All Mu phages used have the *c*ts62 temperature-sensitive repressor mutation so that they can be induced from lysogens (18). A "d" in a phage description indicates defective for plaque formation. All "Mud" phages used in this work are proficient for lysogeny, immunity, and transposition.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages are listed in Table 1. Media composition and general bacterial genetic techniques

Abbreviations: kb, kilobase pair(s); Pro$^+$, proline-independent; Trp$^+$, tryptophan-independent.
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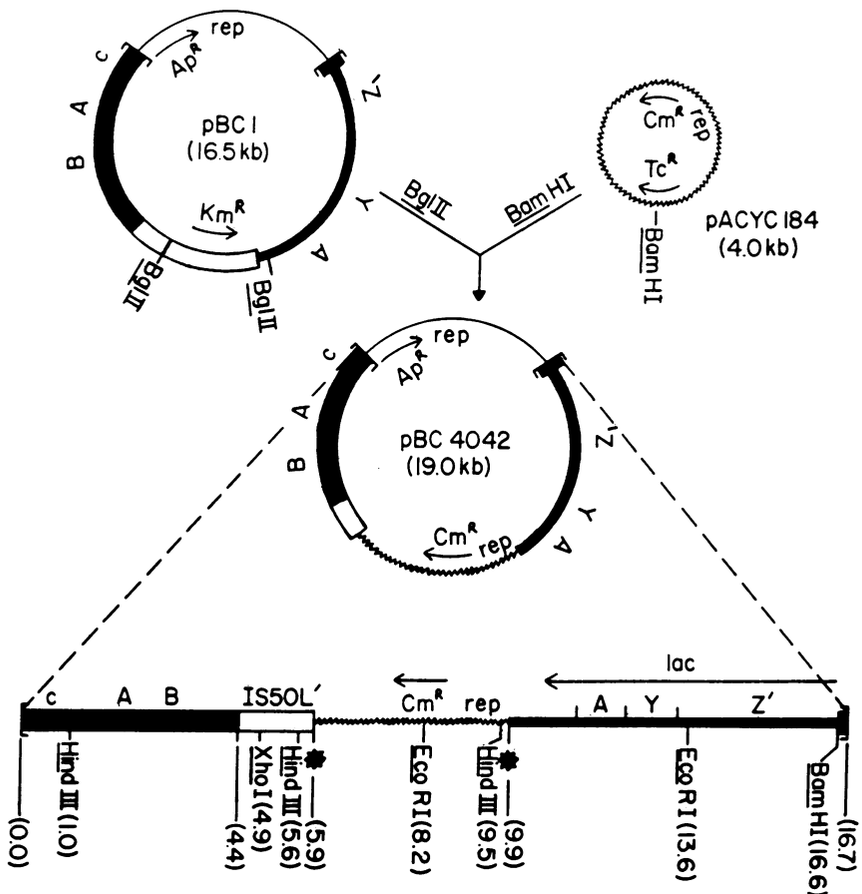

FIG. 1. Construction of Mu-replicon bacteriophage MudII4042. DNA from the P15A plasmid pACYC184 (19) (squiggle line) was cleaved with *Bam*HI and ligated with *Bgl* II-cleaved pBC1 DNA. pBC1 contains an insertion of mini-Mu-*lac* bacteriophage MudII1681 (8), which has only two *Bgl* II sites. The ligated DNA was used to transform the Mu-immune cell M8820Mucts, and ampicillin- and chloramphenicol-resistant transformants were selected and screened for kanamycin and tetracycline sensitivity. The *Bgl* II fragment lost from MudII1681 contains the kanamycin resistance gene, and insertion in the *Bam*HI site of pACYC184 inactivates the tetracycline resistance gene. The resulting Mu construction MudII4042 (lower line) on the plasmid pBC4042 has the Mu left end with the Mu repressor *c* and the transposition-replication genes *A* and *B*, and also has 117 base pairs from the right end of Mu (filled bar), so that it is proficient for intracellular transposition. A nontransposable segment from the IS50L arm of Tn*5* that was next to the kanamycin resistance gene on MudII1681 is also present (open bar). An * indicates the location of the *Bam*HI and *Bgl* II sites present in the original plasmids and destroyed when ligation to form plasmid pBC4042 took place.

have been described (22). Procedures for handling bacteriophage Mu were described (23). In general, bacteriophage Mucts were stored and grown as lysogens and induced by heating, and the resulting lysates were used to transduce recipient cells with selection for drug resistance and either auxotrophic complementation or carbon source utilization. Ampicillin and chloramphenicol were used at concentrations of 25 μg/ml. General DNA manipulation and cloning procedures have been described (24).

RESULTS

The Mu-replicon bacteriophage MudII4042 was constructed by introducing a plasmid replicon into the mini-Mu-*lac* element MudII1681 (8), as described in Fig. 1. The replicon and a selectable gene for chloramphenicol resistance were from the pACYC184 plasmid cleaved with *Bam*HI. The *lac* operon segment from MudII1681 is missing the *lac* operon promoter and the translation initiation site and first eight amino acid codons of *lacZ*. It is positioned 117 base pairs from the right end of Mu such that transcription and translation from outside gene-controlling regions can proceed into the *lacZ* sequence to form hybrid proteins that have β-galactosidase enzymatic activity (7).

The scheme used to clone DNA sequences *in vivo* with MudII4042 is described in Fig. 2. Cells lysogenic for MudII4042 and a complementing Mucts prophage are heated to induce transposition to different sites during phage replication. DNA sequences can become flanked by copies of MudII4042. Packaging starts from the left side of one MudII4042

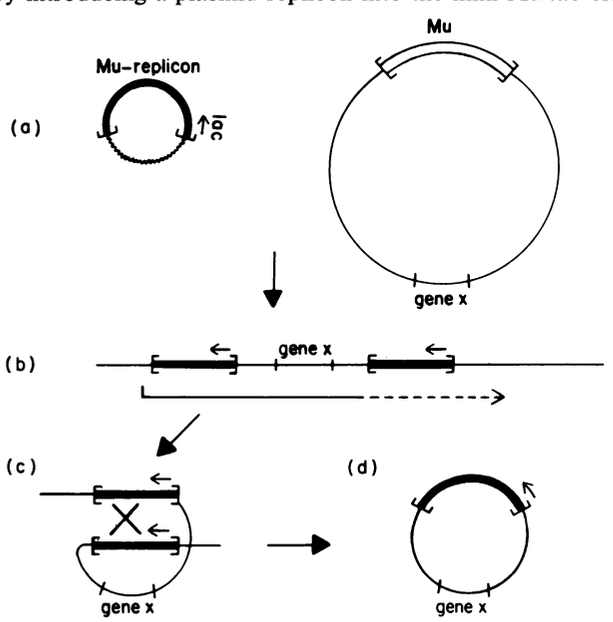

FIG. 2. Cloning with Mu-replicon. (*a*) A plasmid containing a Mu-replicon prophage (filled bar) is introduced into a Mucts (open bar) lysogenic cell by transformation, transduction, or conjugation. The Mu-replicon could either be on a plasmid such as pBC4042 or on a Mu-replicon plasmid clone of a DNA segment. (*b*) When the cells are derepressed for Mu growth an intermediate in the cloning process is presumed to be formed when a copy of the Mu-replicon integrates near a gene *x* and another copy of the Mu-replicon, or a helper Mu prophage, integrates nearby in the same orientation on the other side. Packaging then proceeds by a head-full mechanism from the left side of the Mu-replicon, including the gene *x* sequence and at least a part of the other Mu-replicon or helper Mu, to a total of 39 kb, as underlined with an arrow. (*c*) After infection of a recipient cell, recombination can occur between Mu homologous regions to form a plasmid. (*d*) If the Mu-replicon contains a *lac* gene fusion segment (arrows), genes transcribed in a direction away from gene *x* can form fusions to *lacZ*. In the case of the MudII4042 bacteriophage described in this paper, the *lac* gene fusion must also have a translation initiation signal and codons aligned with *lacZ*, and the resulting hybrid protein must not be lethal to the cell.

and can include bacterial sequences together with the Mu sequences inserted on the other side. After infection of a recipient cell, homologous recombination between Mu sequences can take place to form a plasmid carrying the DNA sequences.

To test this *in vivo* cloning scheme, the pBC4042 plasmid containing the Mu-replicon MudII4042 phage was introduced into M8820Mucts lysogenic cells by transformation and selecting for ampicillin and chloramphenicol resistance. Mu growth was induced, but a complete clearing of the culture was not observed as is seen with the parent strain M8820Mucts. This phenomenon may be due to the presence of the defective phage on a high copy number plasmid. Nevertheless, normal Mu titers of over 10^9 plaque-forming units/ml were obtained. The lysates were used to infect various mutant *E. coli* strains. Chloramphenicol-resistant transductants of XPh43Mucts were obtained at a frequency of $10^{-5.5}$ per plaque-forming unit (Table 2). These transductants are not expected to be due to insertions of MudII4042 in the chromosome, since the presence of the high copy number replicon in the chromosome would be lethal. Thirty-three percent of them were ampicillin resistant, implying that the original pBC4042 plasmid had been transduced. Ampicillin-sensitive transductants were suspected to have picked up chromosomal sequences. Tryptophan-independent (Trp^+) and proline-independent (Pro^+) transductants were found at frequencies of 10^{-2} to 10^{-4} per chloramphenicol-resistant transductant. Plasmid DNA was isolated from seven Pro^+ and two Trp^+ transductants and used to transform the original XPh43Mucts strain. All chloramphenicol-resistant transformants were Pro^+ or Trp^+, and all Pro^+ and Trp^+ transformants were chloramphenicol resistant, implying that both markers were on a plasmid. Similarly, these pACYC184-derived plasmids, which do not have the plasmid partition function *par* (25), were cured at a spontaneous frequency of 50% of the cells in a colony, with the simultaneous loss of both markers.

Pro^+ transductants of XPh43Mucts were examined in more detail. Seventy-five percent of them also contained the *phoA* gene for alkaline phosphatase, as detected on phosphatase indicator plates containing the chromogenic substrate XP (17). XPh43Mucts has a deletion of the entire *brnQ–phoA–proC–phoB* region, so the complementation of the *proC* requirement and the unselected presence of a linked gene implies that these clones do indeed carry the *proC* gene and not some sort of suppressor. Clones of the *phoA* gene (Table 2) were also isolated among chloramphenicol-resistant transductants on XP medium, and 65% of them were found to be Pro^+. Note that these *phoA* clones were identified by their enzymatic activity, without a growth selection. None of 37 $proC^+$ or $phoA^+$ selected clones contained the *brnQ* gene (17), which is also removed by the deletion in strain XPh43Mucts. The levels of phosphatase suggested that the *phoB* gene was not present on any of these clones either (17). Plasmid DNA extracted from several chloramphenicol-resistant Pro^+ and phosphatase-positive transductants of strain XPh43Mucts was examined. Their *Bam*HI, *Bst*EII, *Eco*RI, *Hin*dIII, and *Xho* I restriction digestion patterns were consistent with published maps and sequences of this region (26–29). All the expected internal fragments from MudII4042 were present, and both possible orientations were found. The size of the DNA segments for 12 $proC^+$ clones ranged from 2 to 15 kb, with an average of 6.2 kb, which is consistent with the predicted mechanism described in Fig. 2 (see *Discussion*). The MudII4042 prophage in five different clones were tested genetically for their ability to retranspose and yield new plasmid clones. All could do so, indicating that no Mu sequences had been lost or mutated. As summarized in Table 2, genes from a total of eight different operons were cloned.

The Mu-replicon cloning system was tested with $recA^-$ and nonimmune recipient cells. Chloramphenicol-resistant and auxotroph-complementing transductants could still be obtained with these cells but at 1/100th the frequency in

Table 2. Cloning of *E. coli* operons

Donor cell/plasmid	Recipient	Gene selected	Transductants/pfu	No. cotransductants/ total no. scored
M8820Mucts/pBC4042	XPh43Mucts	*proC*	1.1×10^{-7}	16 $phoA^+$/20
		phoA	5.7×10^{-8}	11 $proC^+$/17
		trp	2.9×10^{-8}	
	DH37Mucts	*trp*	7.1×10^{-9}	
MC4100-3G/pEG176	XPh43Mucts	*proC*	1.1×10^{-6}	
	DH37Mucts	*trp*	1.8×10^{-8}	
	M8834m	*leuABCD*	9.2×10^{-8}	18 $araC^+$/75
		araC	3.9×10^{-8}	
	M8820Mucts	*leu*	1.6×10^{-7}	
		proA,B	8.2×10^{-7}	
XPh43Mucts/pEG109	DH37Mucts	*trp*	1.1×10^{-7}	
		met	2.5×10^{-7}	
	M8834m	*leuABCD*	9.4×10^{-8}	5 $araC^+$/20
		araC	9.8×10^{-8}	
	M8820Mucts	*proA,B*	5.6×10^{-7}	
		araCBAD	1.0×10^{-8}	0 leu^+/5
		leu	4.0×10^{-8}	0 $araCOBAD^+$/18
	MC4100-3G	*arg*	7.5×10^{-6}	

Lysates with titers of 2–6 $\times$ 10^9 plaque-forming units (pfu)/ml were made from the donor cells containing the indicated plasmids. pEG176 is an *arg* clone selected in strain MC4100-3G and pEG109 is a *proC phoA* clone selected in strain XPh43Mucts. Overnight cultures of the recipient cells were adsorbed at a multiplicity of infection of approximately 1 for 20 min. About 10^{-4} to $10^{-5.5}$ chloramphenicol-resistant transductants per plaque-forming unit were obtained after a 1:10 dilution with LB medium and incubation for 60 min at 30°C to allow expression of chloramphenicol resistance. Biosynthetic genes were selected by plating on minimal M63/glucose/chloramphenicol plates supplemented with any additional requirements. *araC* clones were selected on M63/arabinose/chloramphenicol plates supplemented with any additional requirements. *phoA* clones were selected as blue, alkaline phosphatase-containing, colonies on M121/XP/chloramphenicol plates.

recA$^-$ recipients and 1/10th the frequency in nonimmune cells.

Fusions of the *lac* operon to different genes were obtained with MudII4042. Among all chloramphenicol-resistant transductants 0.6% were lactose-utilizing (Lac$^+$) as indicated on lactose MacConkey medium, and among 12 *proC*$^+$ transductants none were Lac$^+$ on MacConkey medium but two expressed low levels of β-galactosidase as indicated by cleavage of the chromogenic substrate XG (22). Two *lac* fusions to a specific promoter, that of the *araBAD* operon, were identified among 38 leucine-independent clones selected in strain M8820Mucts. *araBAD* is transcribed away from *leu*, and *lac* fusions to it are easily scored as being Lac$^+$ only in the presence of the inducer L-arabinose (15, 16).

DISCUSSION

We describe here a convenient system for cloning DNA sequences *in vivo*. A plasmid replicon was inserted into a mini-Mu bacteriophage to allow the replication of DNA sequences linked to it. The plasmids formed have a single copy of the Mu sequences and are stable even in recombination-proficient cells, in contrast to previously described Mu cloning systems, which required two copies of the Mu element to join the cloned sequence to a replicon (1–3). Mu transduction was used to transfer the DNA sequences to be cloned to new cells. The size of the DNA cloned with this system is limited by the amount of DNA that can be packaged inside the Mu head minus the size of the Mu-replicon. The head-full packaging mechanism of Mu can accommodate approximately 39 kb, so that a 16.7-kb MudII4042 phage plus a minimal segment of a second Mu for homologous recombination would allow clones of up to 22.3 kb to be formed. If one assumes that homologous recombination is proportional to length, it can be calculated that the average size of the clone would be 7 kb, which is in close agreement with what was found. This maximal size constraint can be removed by using conjugation to transfer the DNA, as done in previously described Mu cloning systems. The relatively small size and the high copy number of the clones obtained make it convenient to isolate plasmid DNA and to locate a gene on a cloned sequence by comparing restriction digestion patterns of plasmid DNA from a few independent clones.

This system also results in the formation of *lac*-gene fusions that are useful for studying gene expression and function (10, 12, 13). The fusions are located on plasmids that can be isolated, examined *in vitro* to map their gene-controlling elements, and introduced into different genetic backgrounds. Essential genes can be fused to *lac*, since another copy is present in the chromosome. Fusions to genes in a particular region can be isolated among clones of an upstream nearby gene, and fusions to a selected gene itself can occasionally be isolated if they are formed in a nonessential terminal part of the gene or its operon. The Mu-replicon described here, MudII4042, results only in the formation of the hybrid protein type of *lac* fusions. Transcriptional *lac* fusions, which are formed more frequently, can be obtained by making another version of the Mu-replicon-*lac* phage (8).

Additional variations of the Mu-replicon bacteriophage can be made with different replicons and genes for new applications. Larger or smaller Mu-replicons can be used to select clones of different average size. Lower-copy replicons can be used to clone genes that are lethal when present in high copy numbers or to study genes whose regulation is altered when they are present in high copy numbers. Note that the copy number of a DNA sequence cloned with MudII4042 can be reduced to one by using *polA*$^-$ cells, since these cells do not replicate the P15A replicon and selection for maintenance of the plasmid requires its integration in the chromosome. Different replicons, selectable genes, and fusable gene segments can also be used to make Mu-replicons for cloning and fusing genes in other species in which bacteriophage Mu can transpose.

Note Added in Proof. The Mu replicon bacteriophage MudII4042 contains 117 base pairs from the right end of Mu (ref. 7 cites 116). The Mu sequence reported by Kahmann and Kamp (30) is missing 1 base pair at position 31 from the right end according to Allet (31). This change does not affect the ability to form transcript-translation fusions with *lacZ*.

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