Utilization of IncP-1 Plasmids as Vectors for Transposon Mutagenesis in Myxobacteria

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No free plasmid has ever been found in the myxobacterium *Myxococcus xanthus*, but IncP-1 plasmids are able to integrate into the chromosome of this bacterium. The frequency of integration depends greatly upon the structure of the IncP-1 plasmid used. This property has been used to devise new delivery systems for transposon mutagenesis in this species. Plasmids with low integration efficiencies have proved to be efficient donors of Tn5, while plasmids with very high frequencies of integration could be used directly to generate mutations. These vectors have also proved efficient for Tn5 transfer into other species of myxobacteria, which have not so far been susceptible to genetic analysis.

INTRODUCTION

The myxobacterium *Myxococcus xanthus* is a very peculiar Gram-negative bacterium. Its numerous unusual properties have interested biologists who study morphogenesis (Kaiser *et al.*, 1979), gliding motility (Hodgkin & Kaiser, 1979) and protein secretion (Nicaud *et al.*, 1984), as well as biotechnologists who try to use it for antibiotic or protein production (Breton *et al.*, 1984; Gerth *et al.*, 1983; Rosenberg *et al.*, 1984).

Genetic studies of this species started with the discovery of generalized transducing phages (Campos *et al.*, 1978). Later it was found that the coliphage P1 can adsorb and inject its DNA into, but cannot develop in *M. xanthus* (Kaiser & Dworkin, 1975). It was later shown that coliphage P1 allows the transfer of transposon Tn5, which then integrates randomly into the chromosome, generating mutations (Kuner & Kaiser, 1981). More recently this vector has been successfully utilized to transfer *M. xanthus* genes that had been cloned in *Escherichia coli* back into the original host (Shimkets *et al.*, 1983). Only a few strains of *M. xanthus* are, however, susceptible to genetic analysis, although several other strains or species have proved to be of interest, with regard to antibiotic production (Reichenbach *et al.*, 1988) or morphogenesis (Kaiser *et al.*, 1979).

The broad-host-range plasmid RP4 can be transferred by conjugation from *E. coli* to *M. xanthus*, where it is maintained integrated in the chromosome (Breton *et al.*, 1985). The efficiency of the transfer depends on both the recipient strain and the structure of the RP4 derivative used (Jaoua *et al.*, 1987). In some instances it can reach values of 10⁻¹.

We describe here the use of RP4 derivatives to promote efficient transfer and, consequently, transposition of Tn5 into characterized strains of *M. xanthus*, as well as into other strains of the same or other species (*Myxococcus fulvus* and *Myxococcus stipitatus*).

METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used are listed in Table 1.

All three *Myxococcus* species were grown at 30 °C in CYE medium (Zusman *et al.*, 1978). *Myxococcus fulvus* was additionally plated on MXF medium: Casitone (Difco) 0.5%; CaCl₂, 0.05%; MgSO₄·7H₂O, 0.05%; pH 7.2 (K. Gerth, personal communication). *E. coli* was grown at 37 °C in L broth (Miller, 1972).

These media were solidified with 1.2% agar (Biomérieux). Milk plates were prepared from CYE plates overlaid with 3 ml of a 2:1 mixture of soft agar (0.6%) and sterilized skimmed milk. Proteolytic activity was estimated by the size of the lysis halo surrounding colonies toothpicked onto CYE milk plates, as compared to that of control strains. Proteolytic activity and total protein were assayed in the supernatant of liquid cultures as described before (Nicaud *et al.*, 1984).

The antibiotics used and their final concentrations (mg l⁻¹) were: for myxobacteria, kanamycin sulphate (Kan), 75 or 50 (Bristol Laboratories); oxytetracycline chlorohydrate (Otc; used to select strains of *Myxococcus* harbouring a *tet* gene), 12.5 (Roussel Uclaf); and streptomycin sulphate (Str) 500 (Diamant); for *E. coli*, nalidixic acid (Nal), 100; tetracycline (Tet), 10 (Roussel-Uclaf); and phleomycin (Phl), 5 (Civea).

Matings. These were done as described by Breton *et al.* (1985). The donor strains were counterselected with streptomycin. Transfer frequencies are reported as the number of transconjugants per recipient cell.

Plasmid extraction and detection. Plasmids were extracted from *E. coli* by the clear-lysate technique (Cohen & Miller, 1969) and the supercoiled plasmid DNA was separated from linear chromosomal DNA by ultracentrifugation in a CsCl/ethidium bromide gradient (Clewell & Helinski, 1969). Free supercoiled plasmid DNA was visualized in agarose gels after *in situ* lysis (Eckhardt, 1978).

Preparation of DNA and Southern blot hybridization. The blot hybridization method of Southern (1975) was used to determine the size of *M. xanthus* chromosomal DNA fragments displaying homology with either Tn5 or pME305. Chromosomal DNA was extracted as described by Breton *et al.* (1985); 5 µg DNA was digested with a restriction enzyme (*Sal*I or *Pst*I) as described by the supplier (Boehringer-Mannheim and Appligen, respectively). λ DNA digested with *Eco*RI and *Hind*III (Boehringer Mannheim) was used as a standard.

The plasmid DNA to be used as a probe was labelled by nick-translation, using 2 mCi (74 MBq) [α -³²P]dCTP (Amersham) ml⁻¹ and 200 ng DNA in 25 µl volumes incubated at 15 °C. Hybridization of the labelled probe to the chromosomal DNA fragments attached to nitrocellulose was done as described by Breton *et al.* (1985).

Construction, preparation and isolation of an RP4::Tn5 plasmid. Phage λNK467 was used to transfer Tn5 to a culture of *E. coli* W3101 Nal(RP4). Tn5 transposition was monitored by the acquisition of resistance to bleomycin/phleomycin (Mazodier *et al.*, 1985). The RP4::Tn5 plasmids were selected after mating *E. coli* W3101 Rif^r recipients with the pool of Bl^r transduced cells, as Bl^rOc^r transconjugants. One RP4::Tn5 plasmid was purified, after checking that no loss in transferability had occurred, and that its size was as expected.

RESULTS

Transposition of Tn5 in M. xanthus

We have studied the transposition of Tn5 carried by plasmid pME462, which had been shown previously to integrate with a low frequency into the *M. xanthus* chromosome (Jaoua *et al.*, 1987). Two recipient strains were used: DZ1, which is a good conjugal recipient of RP4, and CM207, which is a rather poor recipient (Breton *et al.*, 1985).

In both instances, only two classes of transconjugants were obtained (Table 2). The majority were Km^r only (98% of the CM207 transconjugants and 92% of the DZ1 transconjugants) whereas a minority also expressed Oc^r, which, like Km^r, is encoded by pME462. Increasing the transfer frequency by using an intermediate, non-selected *E. coli* host (tri-parental cross) did not modify these ratios (Table 2).

To check whether the Km^rOc^r transconjugants carried an integrated pME462 in their chromosome, we demonstrated the ability of six randomly chosen transconjugants of this class to retransfer the plasmid to *E. coli*. Then total DNA from one of the transconjugants was digested with *Pst*I and probed with radioactive pME305 DNA. Hybridization showed that the entire plasmid was present (data not shown).

Km^rOc^s transconjugants, however, could result either from Tn5 transposition into the chromosome of a bacterium in which the plasmid had not been integrated, or from a deletion of part of an inserted pME462 (Jaoua *et al.*, 1986). When the DNA from four randomly chosen transconjugants was probed with radioactive pME305 DNA, no hybridization was detected (results not shown). However a dot-blot analysis of a pool of transconjugants of this class with

Table 1. *Bacterial strains and plasmids*

Strain	Relevant characteristics*	Source or reference
<i>Myxococcus xanthus</i>		
DK101	Reference wild-type	Hodgkin & Kaiser (1979)
DZ1	Str ^r	Zusman <i>et al.</i> (1978)
CM207	Str ^r mutant of DK101	Breton <i>et al.</i> (1985)
Mxx12	Natural isolate; Str ^s	Gerth <i>et al.</i> (1983)
CM500	Str ^r mutant from Mxx12	This work
<i>Myxococcus fulvus</i>		
Mxf16	Myxothiazol-producing strain	Gerth <i>et al.</i> (1980)
CM700	Str ^r mutant from Mxf16	This work
<i>Myxococcus stipitatus</i>		
Mxs1	Natural isolate	Provided by Dr Reichenbach, GBF, Braunschweig, FRG
Mxs2	Natural isolate	
CM800	Str ^r mutant of Mxs1	This work
CM850	Str ^r mutant of Mxs2	This work
<i>E. coli</i>		
W3101 Nal	<i>recA13 trpE</i> Nal ^r	Kopecko <i>et al.</i> (1976)
W3101 Rif	<i>recA13 trpE</i> Rif ^r	Kopecko <i>et al.</i> (1976)
C600	<i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</i>	Appleyard (1954)
IncP-1 plasmids		
RP4	Km Tc/Oc Ap	M. Rella via J. Brevet, Université Paris XI, Orsay, France
pME305	Tc/Oc Ap rep ^{ts}	Rella (1984)
pME462	Tc/Oc Ap rep ^{ts} Km Bl (pME305::Tn5)	Rella (1984)
pUZ8	Km Tc/Oc Hg	Hedges & Matthew (1979)
RP4::Tn5	Km Tc/Oc Ap Bl	This work
pCM2019	Km Tc/Oc Hg	Jaoua <i>et al.</i> (1986)
pCM2023	Km Tc/Oc Ap	Jaoua <i>et al.</i> (1987)
Other plasmids		
ColE1::Tn5	Km Bl rep ^{ts}	Gift from C. Elmerich, Institut Pasteur, Paris, France
Phage		
λNK467	<i>b221 rex::Tn5 cI857</i> <i>O29am P80am</i>	N. Kleckner via J. P. Bohin, Université Paris XI, Orsay, France

* Km, Tc/Oc, Ap and Bl (resistance to bleomycin/phleomycin) are plasmid markers.

Table 2. *Frequencies of transposition and of plasmid integration using different vectors and two recipient strains*

The donor strain was *E. coli* W3101 (Nal). The frequencies represent the number of cells resistant to the corresponding antibiotic per recipient Str^r *Myxococcus* cell. In each case 100 colonies from the selection plate were toothpicked on a plate containing the other antibiotic, thus allowing estimation of the percentage of clones displaying only one resistance: (% Oc^s) among the Km^r cells or (% Km^s) among the Oc^r cells.

Recipient strain...	DZ1		CM207	
	Km ^r (% Oc ^s)*	Oc ^r (% Km ^s)	Km ^r (% Oc ^s)*	Oc ^r (% Km ^s)
Vector				
pME462	5×10^{-4} (92)	8×10^{-5} (0)	2×10^{-8} (98)	6×10^{-9} (0)
pME462 with intermediate host†	9×10^{-4} (92)	4.6×10^{-5} (0)	1.3×10^{-7} (95)	—
RP4::Tn5	1.5×10^{-3} (45)	5.3×10^{-4} (0)	10^{-6} (85)	—
pME305 + ColE1::Tn5	1×10^{-8} (100)	7.5×10^{-6} (100)	ND	ND
pCM2019	2×10^{-1}	2×10^{-1}	1×10^{-3}	1×10^{-3}

ND, None detected. —, Not done.

* Estimated from 100 toothpicked Kan^r colonies tested on Otc agar.

† Exponentially growing *E. coli* C600 cells were added to the conjugation mixture at a concentration equal to that of the donor cells.

Fig. 1. Autoradiogram of Southern blot hybridization with a ColE1::Tn5 probe. Total genomic DNA from the test strains was digested with *Sal*I restriction endonuclease and the fragments generated were separated on a 0.7% (w/v) agarose gel. The DNA was transferred to nitrocellulose according to Southern (1975) and hybridized with nick-translated ColE1::Tn5 probe DNA. Lanes 1 to 4, DZ1 (Km^rOc^s) DNA [transconjugants from DZ1 × W3101(pME462) mating]. Lanes 5 and 6, DZ1 (Km^rOc^s) DNA [transconjugants from DZ1 × W3101(pME305, ColE1::Tn5) mating].

the same probe showed some hybridization. In contrast, when probed with radioactive ColE1::Tn5 plasmid, after digestion with *Sal*I, the DNA of these four clones showed two distinct bands. The size of these fragments was different in each case (Fig. 1, lanes 1 to 4), indicating that Tn5 was inserted at a different site in each instance (Tn5 possesses a single *Sal*I site). A phenotypic analysis of 1000 transconjugants from each recipient strain was undertaken. The phenotypic traits scored were chosen so as to permit comparison of the results with those obtained previously with strain DK101 using phage P1::Tn5 (Guespin-Michel *et al.*, 1984), according to the method of Kuner & Kaiser (1981). Table 3 shows that transposon mutagenesis was comparable with both techniques.

Two other vectors were compared for their efficiency of transposition of Tn5 measured only, in these instances, as the frequency of Km^rOc^s transconjugants. (i) An RP4::Tn5 plasmid was prepared as described in Methods. Its frequency of transfer was higher than that of pME462 with both recipient strains, but the ratio of Km^rOc^s transconjugants to Km^rOc^s transconjugants was also higher – especially with strain DZ1 (Table 2). (ii) We attempted to mobilize the ColE1::Tn5 plasmid using pME305 as the conjugative plasmid. No Km^rOc^s transconjugants were obtained, while Km^rOc^s transconjugants of DZ1 were recovered at low frequency (Table 2).

Two Km^rOc^s transconjugants of DZ1 were analysed by Southern hybridization of *Sal*I-restricted total DNA with a radioactive ColE1::Tn5 probe. The results showed that Tn5 was integrated at a different place in the chromosome in each case (Fig. 1, lanes 5 and 6).

Insertion of pCM2019 in M. xanthus

Plasmid pCM2019 was obtained after transfer of integrated plasmid pUZ8 in strain DZ1 back to *E. coli* (Jaoua *et al.*, 1987). Its frequency of chromosome integration in *M. xanthus* is very high, even in strain CM207 (Table 2). A total of 2100 transconjugants from strain DZ1 were screened for mutagenesis using the same criteria as were employed for Tn5 mutagenesis. Table 3 shows that similar rates of mutation were obtained in all cases, suggesting that pCM2019 has several possible insertion sites in the chromosome of this strain.

Tn5 transfer into other myxobacterial strains

Several myxobacterial strains have been tested as recipients in matings with the above-described *E. coli* strains. Some of them proved difficult to cultivate under our standard conditions, so we eventually selected four strains belonging to three different species, in order to

Table 3. Frequency of mutations obtained in *M. xanthus*, using different vectors

The donor strains were *E. coli* W3101 Nal harbouring the different plasmids except when the P1::Tn5 phage lysate was used. Transfer frequencies are estimated as described in Table 2. Mutation frequencies are reported as the number of mutated cells for a given trait, per assayed transposed clone (1000 to 2500 depending on the experiment).

Recipient strain	DZ1	CM207	DK101*	DZ1
Vector	pME462	pME462	P1::Tn5	pCM2019
Transposition or transfer frequency	$5 \times 10^{-4}\dagger$	$2 \times 10^{-8}\dagger$	2×10^{-8}	2×10^{-1}
Frequency of mutations to:				
Mot ⁻ ‡	—	8×10^{-3}	1×10^{-3}	—
Prt§	1.1×10^{-2}	1.8×10^{-2}	1.6×10^{-2}	0.9×10^{-2}
Distribution of Prt mutations :				
Prt [±]	2	0	3	7
Prt ⁺⁺	2	2	0	2
Exc [±]	1	3	3	4
Exc ⁺⁺	1	3	2	1

* Results from Guespin-Michel *et al.* (1984).

† Estimated as the frequency of Km^rOc^s clones.

‡ Mutants completely deprived of motility were detected as smooth-edged colonies.

§ Frequency of transconjugant colonies which, on milk agar, were surrounded by a halo of different size from that of the control.

|| The Prt mutants were isolated and purified. Liquid cultures were assayed for both proteolytic activity and total extracellular protein production. Prt⁺⁺ and Prt[±] are hyper- or hypoproteolytic strains, respectively. Exc⁺⁺ and Exc[±] are hyper- or hyposecretors of total protein, respectively.

compare the behaviour of the vectors. Spontaneous mutants to streptomycin resistance were selected from each strain, and each was used as a recipient for the mating.

When RP4::Tn5 and pME462 were used as conjugative plasmids, stable Km^r transconjugants were obtained, but no transconjugants appeared on the Otc plates (Table 4). To check whether this was due to transposition of Tn5 alone, or to a failure of Otc, as the selective agent, to detect the presence of an integrated IncP-1 plasmid in these strains, we used RP4 and pCM2019 (or pCM2023) as conjugative plasmids. No transconjugants were detected with RP4, whereas pCM2019 or pCM2023 yielded Km^r and Oc^r transconjugants with strains CM500, CM800 and CM850. With strain CM700 we obtained some Km^r transconjugants with RP4 when MXF medium was used, but we were unable to detect any other resistance conferred on the strain by this plasmid or by pCM2023 (we tested different concentrations of oxytetracycline, tetracycline or carbenicillin). Dot-blot hybridization of the DNA from 24 Km^r transconjugants with radioactive RP4 showed no hybridization in 16 instances (data not shown), suggesting that 2/3 of the transconjugants have genuine Tn5 transpositions. As with the results obtained with the first *M. xanthus* strains, DZ1 and CM207, no free plasmids were detected in any of the transconjugants analysed.

DISCUSSION

Our results show that several IncP-1 plasmids may be used as delivery systems for transposon mutagenesis in standard strains of *M. xanthus*, even though these plasmids are not 100% suicide vectors.

The transposition frequencies obtained depended on the vector and on the recipient strain. With strain DZ1, not only was the frequency of recovery of transconjugants higher than in strain CM207, but so also was the ratio of integration (Km^rOc^r) to transposition (Km^rOc^s) when both RP4::Tn5 and pME462 were used. This suggests that both the frequency of transfer and the frequency of integration of the transferred plasmids were greater in strain DZ1 than in strain CM207.

Plasmid pME462 was less efficiently integrated than RP4::Tn5 in both strain DZ1 and strain CM207. This effect has been shown to correspond to the deletion of a site on RP4::Tn5 for preferential insertion (Jaoua *et al.*, 1987). Thus pME462 can be considered a good

Table 4. Frequencies of transconjugants obtained using four different *Myxococcus* strains as recipient

Recipient strain	Plasmid	Transconjugant frequencies on selective media*	
		Kan Str	Otc
<i>M. xanthus</i> CM500	RP4::Tn5	1×10^{-7}	$< 1 \times 10^{-8}$
	pME462	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$
	RP4	$< 3 \times 10^{-7}$	$< 3 \times 10^{-7}$
	pCM2019	1.2×10^{-6}	7×10^{-7}
	(or pCM2023)†		
<i>M. stipitatus</i> CM800	RP4::Tn5	3.2×10^{-7}	$< 5 \times 10^{-8}$
	pME462	6.5×10^{-7}	$< 5 \times 10^{-8}$
	RP4	$< 3 \times 10^{-8}$	$< 3 \times 10^{-8}$
	pCM2019	5×10^{-7}	5×10^{-7}
	(or pCM2023)†		
<i>M. stipitatus</i> CM850	RP4::Tn5	1.8×10^{-7}	$< 1 \times 10^{-7}$
	pME462	2.2×10^{-7}	$< 1 \times 10^{-7}$
	RP4	$< 3 \times 10^{-8}$	$< 3 \times 10^{-7}$
	pCM2019	2×10^{-7}	2×10^{-7}
	(or pCM2023)†		
<i>M. fulvus</i> CM700‡	RP4::Tn5	2×10^{-8} (2×10^{-6})	$< 1 \times 10^{-8}$ ($< 1 \times 10^{-8}$)
	pME462	2×10^{-7} (5×10^{-6})	$< 1 \times 10^{-8}$ ($< 1 \times 10^{-8}$)
	RP4	$< 2 \times 10^{-8}$ (9×10^{-8})	$< 2 \times 10^{-8}$ ($< 9 \times 10^{-9}$)
	pCM2019	$< 2 \times 10^{-8}$ (7×10^{-4})	$< 2 \times 10^{-8}$ ($< 7 \times 10^{-8}$)
	(or pCM2023)†		

* *E. coli* W3101 Nal was the donor in each case. Frequencies are the means of two or three estimations. In some cases, unstable transconjugants appeared on the Kan Str selection plates, but were not viable when toothpicked on fresh medium; these are not reported here. The concentrations of antibiotics were (mg l^{-1}): Kan, 50 or 75; Str, 500; Otc, 8 or 12.5. For Kan and Otc the results obtained with both concentrations were identical and are accordingly reported together.

† In some cases pCM2023, which is another high frequency transfer vector with regard to DZ1 and CM207, was used instead of pCM2019. Since the results were similar, they are reported together.

‡ The results in parentheses are those obtained when selective and numeration plates were made with MXF medium.

transposition vector for the two strains studied here. When the frequency of transfer was increased (for instance by using an intermediate, non-selected *E. coli* host) the ratio of the two integration events did not change, and satisfactory transposition frequencies were obtained, even with strain CM207. The use of pME305 to mobilize plasmid ColE1::Tn5 into *M. xanthus*, although demonstrated, was not efficient. By contrast, pCM2019, which can be considered a high efficiency of integration plasmid in strains DZ1 and CM207, was shown to insert in several sites in the chromosome of strain DZ1, thus generating mutations similar to those obtained with Tn5.

Other myxobacterial strains were used as recipients, and transfer of some of the plasmids to four of them was achieved. Tn5 transposition occurred in these recipient strains, although at lower frequencies than those obtained for the two standard strains. Thus, gene transfer from *E. coli* has now been achieved with several myxobacterial strains belonging to three different species, which widens the range of genetic studies possible with myxobacteria and encourages the testing of the IncP-1 vectors with other members of this group. The fate of the conjugated plasmids might not be identical in all strains, however. So far, we find that only pCM2019 and pCM2023 are maintained in all four new strains. These plasmids, isolated after retransfer to *E. coli* from the chromosome of strain DZ1 have a modification(s) which confers on them both a high efficiency of integration in strains DZ1 and CM207 and a low, but measurable, efficiency of transfer in the other strains, including strain Mxx12 of *M. xanthus*.

Work is in progress in our laboratory to elucidate both the mechanism of recombination involved during the integration of RP4 into the chromosome of myxobacteria, and the differences displayed by the strains studied with respect to that integration.

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