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Transposon Mutagenesis in *Caulobacter crescentus*

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Transposons Tn*5* (Km) and Tn*7* (Tp and Sm) were transferred to *Caulobacter crescentus* via P-type antibiotic resistance factors. Transposition was demonstrated by the isolation of chromosomal insertions of each transposon. With *C. crescentus* strains harboring RP4 *aphA*::Tn*7*, the introduction of a wild-type RP4 resulted in the loss of the resident plasmid. Simultaneous selection for Km^r and Sm^r yielded colonies with chromosomal insertions of Tn*7*. Examination of over 10,000 chromosomal insertions of Tn*7* indicated no auxotrophic or motility mutants. Thus, Tn*7* appears to have a high specificity of insertion in *C. crescentus*. The Mu-containing plasmid pJB4JI transferred Tn*5* to *C. crescentus*, but the plasmid was not maintained. Control experiments showed that recovery of Mu-containing plasmids occurred at very low frequencies in *C. crescentus* and that the plasmids which were recovered had undergone extensive deletion of plasmid DNA. Presumably, some part of the Mu genome was not tolerated by *C. crescentus*. The instability of the Mu-containing plasmids makes them excellent vectors for the introduction of transposons, and we have used pJB4JI to isolate chromosomal insertions of Tn*5*. When several thousand of these insertion mutants were examined, we found auxotrophic and motility mutants at frequencies of 1 and 2%, respectively. These results indicate that Tn*5* had a low specificity of insertion in *C. crescentus* and therefore would be a useful mutagen for obtaining a variety of mutant phenotypes.

The recent construction of a genetic map (18; J. Barrett et al., manuscript in preparation) provides new opportunities for the genetic analysis of differentiation in the dimorphic bacterium *Caulobacter crescentus*. These opportunities would be enhanced by the availability of selectable mutations, such as those attributable to the insertion of a transposon. To date, naturally occurring transposons have not been identified in *C. crescentus*. However, we have shown that transposons can be introduced by P-type antibiotic resistance factors and that two of these transposons, Tn*5* (Km) and Tn*7* (Tp and Sm), cause chromosomal insertions (7). These results indicate that transposition does occur in *C. crescentus* (7).

To isolate large numbers of mutants containing chromosomal insertions, we needed an efficient method to eliminate the vector used to introduce the transposon. One such method is to eliminate a resident plasmid by the introduction of another plasmid of the same incompatibility group. This method was used successfully to eliminate an RP4 plasmid containing Tn*7*, and mutants were isolated in which Tn*7* had transposed to the *C. crescentus* chromosome (7). A second approach is to introduce transposons on a vector that is not maintained by the host. This approach has been used with various bacteriophages which infect *Escherichia coli* or *Salmonella typhimurium* (16) and with P1 and *Myxococcus xanthus* (17). Beringer et al. (3) have constructed a P-type plasmid, pJB4JI, containing both the bacteriophage Mu and the transposon Tn*5*. When this plasmid is transferred to a bacterium such as a *Rhizobium* species, the plasmid is not maintained by the recipient bacterium, owing to an undetermined property of Mu. Selection for kanamycin resistance results in the selection of cells which have Tn*5* inserted in the recipient chromosome. We have used both of these methods to obtain a large number of insertion mutations in *C. crescentus*.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Strains harboring Mu-containing plasmids were unstable and were stored in the presence of 10% dimethyl sulfoxide at −76°C to maintain donor ability.

Bacterial matings. Bacterial matings were performed as described by Ely (7). Crosses between *E. coli* strain 1830 and *C. crescentus* recipients were plated on medium supplemented with 50 μg of kanamycin per ml to select for Tn*5* and 200 μg of streptomycin per ml to select against the donor. Transductions were performed by using the generalized transducing phage

TABLE 1. Bacterial strains

Strain	Genotype[a]	Source, derivation, or reference
C. crescentus		
CB15	Wild type	This laboratory
SC392	*proA103*	Spontaneous in CB15
SC545	*lysA103*	Spontaneous in CB15
SC859	*proA103 str-140*	Str in SC392
SC1004	*str-152*	Str in CB15
E. coli		
J53(RP4)	*pro met* (RP4 *bla aphA tet*)	H. M. Meade[b]
JC5466(RP4::Mu)	*trp his recA56 spc rif* (RP4::Mu cts62)	4
2174	*pro met* (pPH1JI)[c]	3
1799E	*cys* (pPH1JI::Mu [pJB9JI])	3
1830	*pro met* (pPH1JI::Mu::Tn*5* [pJB4JI])	3
GV100(pGV5010)	(RP4::Mu cts61 [::Tn*7*][d] *bla aphA tet*)	M. Van Montagu[b]
hisF860	*hisF860*	11
W3110T$^-$(pRP33)	*thy* (RP4 *bla aphA*::Tn*7 tet*)	2

[a] *bla*, Ampicillin resistance; *aphA*, kanamycin resistance; *str*, streptomycin resistance; *tet*, tetracycline resistance.

[b] H. M. Meade, Harvard University; M. Van Montagu, Rijksuniversiteit, Gent.

[c] pPH1JI is an incP1 plasmid encoding gentamicin, spectinomycin, and low-level streptomycin resistance.

[d] Tn*7* encodes trimethoprim and streptomycin resistance.

φCr30 (9). Plasmids were examined by agarose gel electrophoresis as described by Eckhardt (6).

RESULTS

Tn*7* mutagenesis of *C. crescentus*. We have shown previously that chromosomal insertions of Tn*7* can be isolated in *C. crescentus* after the elimination of RP4 *aphA*::Tn*7* by the introduction of wild-type RP4 (7). We used this procedure to isolate more than 10,000 mutants with chromosomal insertions of Tn*7*. However, when these mutants were examined for the presence of auxotrophic or motility mutants, none were detected. Since large numbers of auxotrophic and motility mutants representing nearly 100 different genes had been isolated previously by enrichment procedures (13, 14; unpublished data), we concluded that as in *E. coli*, Tn*7* did not transpose into the *C. crescentus* chromosome with a low site specificity. Therefore, Tn*7* was not useful for the generation of mutations in a variety of genes.

Transfer of R factors containing bacteriophage Mu to *C. crescentus*. Bacteriophage Mu causes plasmids to be unstable in *Rhizobium* spp. (3). To determine if a similar phenomenon occurs in *C. crescentus*, we examined the transfer of the Mu-containing plasmids to *C. crescentus*. *E. coli* strains harboring either of two Mu-containing derivatives of the P-type plasmids, RP4 (Km, Tc, Ap) and pPH1JI (Gm), were mated with *C. crescentus* strains and plated on media containing the antibiotics shown in Table 2. For comparison, similar matings were performed with strains lacking bacteriophage Mu. Both P-type plasmids were transferred from *E. coli* to *C. crescentus* at a frequency of approximately 100% when Mu was not present (Table 2). However, when Mu was present, the rate of recovery of antibiotic-resistant colonies was reduced by several orders of magnitude.

When a strain containing RP4::Mu cts61(::Tn*7*) was used to transfer streptomycin resistance, colonies were obtained at a frequency of 10^{-5}. Examination of the other plasmid-encoded antibiotic resistances revealed that all

TABLE 2. Transfer of Mu-containing R factors to *C. crescentus*[a]

Donor	Selection	Recovery of antibiotic-resistant colonies
J53(RP4)	Tc	10^0
JC5466(RP4::Mu cts62)	Tc	10^{-8}
J53(RP4)	Km	10^0
JC5466(RP4::Mu cts62)	Km	10^{-6}
GV1000(RP4::Mu cts61 [::Tn*7*])	Sm	10^{-5}
2174(pPH1JI)	Gm	10^{-1}
cys$^-$(pPH1JI::Mu)	Gm	$<10^{-8}$

[a] Stationary-phase cultures of donor strains were mixed with equal volumes of stationary-phase cultures of *C. crescentus* strain CB15, SC859, or SC1004, filtered, incubated for 2 h, and then spread on selective medium after appropriate dilution. Transfer frequencies are the number of antibiotic-resistant colonies per recipient cell in the initial mating mixture. All values are the average of two or more determinations. Antibiotic concentrations were 3 μg of tetracycline (Tc) per ml, 50 μg of kanamycin (Km) per ml, and 50 μg of gentamycin (Gm) per ml.

of the colonies were resistant to tetracycline but only 5% were resistant to kanamycin. Agarose gel electrophoresis revealed that plasmids from representative colonies were considerably smaller than the original plasmid, suggesting that extensive deletion had occurred. Van Vliet et al. (20) have shown that Mu is inserted near the gene for kanamycin resistance when the RP4::Mu *c*ts61 plasmid is formed. Thus, most deletions removing Mu would be expected to remove the gene for kanamycin resistance. Similar results have been obtained when *Agrobacterium tumefaciens* was used as a recipient (20).

When strains containing the pPH1JI::Mu were used as donors, gentamicin-resistant colonies occurred at a frequency of less than 10^{-8}. Thus, the Mu insertion is probably located near the gene for gentamicin resistance or near a plasmid gene necessary for plasmid replication. Since this plasmid rarely gives rise to stable gentamicin resistance in *C. crescentus* and does not carry genes for kanamycin or tetracycline resistance, it is more practical to use as an unstable plasmid for transposon mutagenesis than are the RP4 plasmids.

Effect of the transfer of Mu on recipient cell survival. The reduction of the transfer frequency of a Mu-containing plasmid could be due to the killing of recipient cells, the restriction of incoming DNA, or interference with either plasmid transfer or replication. To determine which of these explanations is appropriate, we examined recipient cell viability in a number of different crosses (Table 3). In crosses in which Mu was not present, recovery of recipient cells after incubation with the donor was similar to that obtained when no donor was present. When a Mu-sensitive *E. coli* strain was used as a recipient, viability was reduced only when Mu was present on the donor plasmid. Presumably, zygotic induction of the Mu prophage resulted in the killing of the recipient cells. When *C. crescentus* was used as a recipient, approximately 20% of the recipient cells survived regardless of the presence of Mu on the donor plasmid. Other experiments demonstrated that this reduction of viability was independent of the presence of an R factor and therefore was due to some property of *E. coli* (data not shown). Thus, in *C. crescentus*, the reduced recovery of antibiotic-resistant colonies was not due to the killing of the recipient cells by zygotic induction of Mu prophage. Since R factors containing segments of *E. coli* chromosomal DNA are transferred at a high frequency from *E. coli* to *C. crescentus* (J. Barrett and B. Ely, unpublished data), it is not likely that restriction of incoming DNA prevents the establishment of incoming Mu-containing plasmids. Therefore, we conclude that some property of the Mu prophage is responsible for our failure to observe the transfer of Mu-containing plasmids to *C. crescentus*.

Use of pJB4JI to introduce the Tn*5* transposon into *C. crescentus*. Since the transfer of pPH1JI from *E. coli* to *C. crescentus* occurred at a high frequency, and since the presence of Mu does not result in the killing of the recipient cells, it is likely that the Mu-containing plasmid was transferred to but not maintained in *C. crescentus*. Such a plasmid would make an excellent vehicle for the introduction of a transposon. This method has been used successfully for *Rhizobium* spp. (3, 5), *A. tumefaciens* (10, 20), and *Salmonella typhimurium* (T. Melton, personal communication). When strain 1830 containing pJB4JI (pPH1JI::Tn*5* Gm Km) was mated with a streptomycin-resistant *C. crescentus* strain, Sm^r, Km^r colonies arose at a frequency of 10^{-6}. Kanamycin resistance was stably maintained in these strains, and representative mutants were shown by agarose gel electrophoresis to be free from plasmids. Since none of these colonies was resistant to gentamicin, we presumed that the plasmid containing Tn*5* and gentamicin resistance had been transferred to *C. crescentus* and that Tn*5* had transposed to the *C. crescentus*

TABLE 3. Effects of the transfer of Mu on recipient cell survival[a]

Donor	Recipient	Selection	Recipient survival (%)	Transfer frequency
J53(RP4)	*E. coli*	Tc	100	10^{0}
JC5460(RP4::Mu)	*E. coli*	Tc	6	10^{-3}
2174(pPH1JI)	*E. coli*	Gm	100	10^{-1}
1799E(pPH1JI::Mu)	*E. coli*	Gm	40	10^{-4}
J53(RP4)	*C. crescentus*	Tc	20	10^{0}
JC5466(RP4::Mu)	*C. crescentus*	Tc	20	10^{-7}
2174(pPH1JI)	*C. crescentus*	Gm	20	10^{-1}
1799E(pPH1JI::Mu)	*C. crescentus*	Gm	20	10^{-8}

[a] Experiments were performed as described in footnote *a* of Table 2. Strain *hisF860* was used as the *E. coli* recipient; SC1004 was used as the *C. crescentus* recipient. In experiments with *E. coli* recipients, tetracycline was added at a concentration of 15 μg/ml. Antibiotic concentrations and abbreviations are given in footnote *a* of Table 2. Transfer frequencies are the number of antibiotic-resistant colonies per recipient cell in the initial mating mixture.

chromosome before loss of the plasmid. Furthermore, we showed that Km^r could be transferred from one strain to another by φCr30-mediated transduction at frequencies comparable to those obtained for chromosomal markers. When similar experiments were performed with φCr30 grown on a strain harboring RP4, transfer of the plasmid-encoded Km^r was undetectable (7). Therefore, we conclude that the Km^r colonies from crosses with 1830 resulted from the insertion of Tn*5* into the *C. crescentus* chromosome.

To optimize conditions for Tn*5* transposition in *C. crescentus*, we analyzed several parameters for their effects on transposition frequency. When temperature was varied from 23 to 33°C, no significant differences were found in the recovery of Km^r colonies, suggesting that Tn*5* transposition in *C. crescentus* is not affected by temperature. Therefore, further experiments were carried out at our standard growth temperature, 33°C. The time of incubation on a filter containing the mating mixture was varied and found to be optimal at 2 to 4 h. Cell numbers were monitored in these experiments and found to remain relatively constant for the first 4 h. Therefore, we concluded that significant growth did not occur during a 4-h incubation on the filter, and consequently, that every Km^r colony represented an independent transposition event. The growth phase of the donor and recipient cultures did not seem to matter, so overnight cultures were used routinely as a matter of convenience.

Analysis of mutants obtained by Tn*5* mutagenesis. Several thousand Km^r mutants were isolated from crosses in which pJB4JI was introduced into a *C. crescentus* recipient. Approximately 1% of these Km^r mutants were auxotrophic, and approximately 2% had altered motility. The auxotrophic mutants were analyzed to determine their nutritional requirement. Results of these analyses are summarized in Table 4. Reversion frequencies ranged from 10^{-5} to less than 10^{-9} for individual auxotrophs. These mutants were stable during genetic transfer, since Km^r and the auxotrophic mutation caused by insertion of Tn*5* cotransferred 100% of the time in transduction experiments. Transductional analyses revealed a number of loci that had not been found among the 300 spontaneously derived mutants studied previously (9; B. Ely et al., unpublished data). Conversely, many loci identified previously were not found among our collection of transposon mutants. These results suggest that Tn*5* can insert in a large number of chromosomal sites in *C. crescentus*.

In addition to identifying auxotrophs and motility mutants, we used our collection of Tn*5* insertion mutants as a source of other kinds of mutations. For example, screening for susceptibility to penicillin resulted in the identification of a mutant with a Tn*5* insertion in the chromosomal gene encoding the *C. crescentus* penicillinase. Similarly, we identified mutants unable to use particular sugars as carbon sources, mutants resistant to particular bacteriophages, and mutants resistant to amino acid inhibition. From the aforementioned experiments, we were able to determine that the frequency of finding a transposon inserted in any particular gene was about 1 in 3,000. Similar results have been obtained for another species of *Caulobacter*, *C. leidyii* (P. Schoenlein, unpublished data). Detailed analyses of all of these mutants will be presented elsewhere.

Localized mutagenesis with φCr30 grown on pools of Tn*5* insertion mutants. The generalized transducing phage φCr30 was grown on pools of Km^r mutants isolated after Tn*5* mutagenesis. These phage were used to transduce a lysine auxotroph to prototrophy with simultaneous selection for Km^r. The resulting colonies were obtained at a low frequency and resulted from cells which had obtained Tn*5* insertions along with the wild-type lysine gene. Bacteriophage φCr30 grown on nine of these colonies showed transductional linkages to *lysA* that ranged from 10 to 60%. Thus, this method can be used to isolate Tn*5* insertions in the vicinity of the *lysA* gene and should be useful for any gene with a selectable phenotype.

DISCUSSION

When a plasmid-containing bacteriophage Mu is introduced to a Mu-sensitive enteric bacterium, zygotic induction occurs and the recipient cell does not survive. In contrast, when the same plasmid is introduced into a Mu-resistant bacterium such as *C. crescentus*, killing does not occur, but Mu prevents the establishment of the plasmid in the recipient cell. Similar results have been obtained with *Rhizobium* spp. (3, 5), *A. tumefaciens* (10, 20), and *Salmonella typhimurium* (T. Melton, personal communication) but not in *Rhodopseudomonas capsulata* (21). The reason for this phenomenon is not known. It is possible that replication of Mu is initiated but is aborted at some intermediate step, owing to the lack of a necessary host function. This aborted replication would leave the plasmid DNA in a form that could not be replicated by the normal host replicative machinery and thus would result in loss of the plasmid. Since Mu is thought to replicate by transposition (12), this type of explanation would suggest a difference in the role of host functions in Mu transposition, as opposed to Tn*5* and Tn*7* transpositions, which occurred successfully in *C. crescentus*.

TABLE 4. *C. crescentus* auxotrophs derived by transpositions of Tn*5*

Nutritional requirement	Mutant locus (no. of mutants)[a]	Reversion frequency[b]
Arginine	*argA* (1)	10^{-6}
	argB (2)	10^{-7}, $<10^{-8}$
	argG (1)	10^{-7}
Cysteine	*cysA* (2)	10^{-6}, 10^{-5}
	cysB (7)	10^{-8} (1), 10^{-6} (2), 10^{-5} (1)
	cysC (1)	10^{-7}
	cysD (2)	10^{-8}
	Unidentified (1)	10^{-5}
Histidine	*hisA* (1)	10^{-7}
	hisB (3)	10^{-7} (2), 10^{-8}
	hisC (1)	10^{-7}
	hisD (3)	$<10^{-8}$ (2), 10^{-7}
	hisF (1)	10^{-7}
	hisG (1)	10^{-8}
	Unidentified (2)	10^{-6}, $<10^{-8}$
Leucine	*leuA* (3)	10^{-6} (2), $<10^{-8}$
Methionine	*metB* (3)	10^{-6}, 10^{-7}, $<10^{-8}$
	metD (1)	10^{-7}
	metF (3)	$<10^{-8}$, 10^{-8}, 10^{-6}
Phenylalanine	*pheA* (1)	10^{-8}
	Unidentified (2)	10^{-6}, 10^{-5}
Purine	*purA* (1)	10^{-6} (1)
	purE (3)	10^{-8} (2), 10^{-6} (1)
Serine	*serA* (3)	10^{-8} (3)
	Unidentified (1)	10^{-7}
Tryptophan	*trpA* (3)	10^{-7}, $<10^{-8}$ (2)
	trpB (4)	$<10^{-8}$, 10^{-8} (2), 10^{-7}
	Unidentified (1)	10^{-7}
Unidentified	(24)	$<10^{-8}$ (2), 10^{-8} (5), 10^{-7} (5)

[a] Mutant loci were determined by transductional analyses with data mutants of the same phenotype from previous studies (9; Ely et al., unpublished data). Analyses of transductional data were performed as described by Ely et al. (8).

[b] Number in parentheses indicates the number of mutants showing the indicated reversion frequency.

Beringer et al. (3) have shown that strains harboring pJB4JI fail to produce viable Mu bacteriophage, suggesting that Tn*5* has been transposed into Mu. Therefore, chromosomal insertion of Tn*5* in *C. crescentus* could occur by any one of three mechanisms: (i) transposition of Tn*5* alone, (ii) transposition of Mu containing Tn*5*, or (iii) insertion of all or part of the pJB4JI plasmid. Integration of the entire pJB4JI plasmid is not likely, since none of the Km^r colonies was resistant to gentamicin. Analysis of auxotrophic mutants obtained after matings with pJB4JI indicated that most of the mutations in these strains were capable of reverting to prototrophy. Since mutations attributable to insertion of Mu would not be expected to revert (15), it is likely that few, if any, of these mutations are caused by the insertion of Mu. Thus, the simplest explanation for the insertion of Tn*5* into *C. crescentus* is Tn*5* transposition.

We obtained large numbers of chromosomal insertions with both Tn*7* and Tn*5*. However, no auxotrophic or motility mutants resulted from transposition of Tn*7* to the *C. crescentus* chromosome. Thus, Tn*7* appears to have a high specificity of insertion in *C. crescentus*, as it does in *E. coli* (1). In contrast, Tn*5* transposition gave rise to 1% auxotrophs and 2% motility mutants, suggesting a relatively low specificity of insertion. Similar results have been obtained

with Tn*5*-induced auxotrophs in *E. coli* (19). These results suggest that transposition specificity is independent of the host species.

We have shown that Mu-containing plasmids are an efficient way to introduce transposons to a Mu-resistant gram-negative bacterium. Others have shown that the method is applicable to other Mu-resistant gram-negative bacteria such as *Rhizobium typhimurium* (3, 5), *A. tumefaciens* (10), and *Salmonella* spp. (Melton, personal communication). The applicability of this system to other bacteria is readily determined by a comparison of the transfer frequencies of P-type plasmids with and without Mu to a Mu-resistant recipient. If the transfer of the plasmid lacking Mu is relatively efficient and the transfer frequencies differ by a factor of 10^6, the Mu-containing plasmid should be an efficient vector for the introduction of transposons.

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