Use of P-1 Incompatibility Group Plasmids to Introduce Transposons into *Pseudomonas solanacearum*

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ABSTRACT

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Strain K60 of *Pseudomonas solanacearum* cannot stably maintain P-1 incompatibility group plasmids. However, it is possible to isolate derivatives that receive and maintain these plasmids. This system has been used to mutagenize the strain with transposons Tn5, Tn7, and Tn10. In *P. solanacearum*, Tn5 and Tn10 are transposed with a relatively high frequency (5×10^{-6}), and 1% of the transposon-harboring clones obtained were auxotrophs. The transposition frequency with Tn7 is low.

RÉSUMÉ

La souche K60 de *Pseudomonas solanacearum* n'acquièrt pas de façon stable les plasmides du groupe P-1. A la suite de différents traitements il est possible d'isoler des dérivés qui reçoivent et maintiennent ces plasmides. Ce système a été utilisé pour mutagéniser cette souche avec les transposons Tn5, Tn7, et Tn10. Dans *P. solanacearum* Tn5 et Tn10 se transposent à relativement haute fréquence (5×10^{-6}) et 1% des mutants obtenus sont auxotrophes. Dans le cas de Tn7 au contraire la fréquence de transposition observèe est trés faible.

Transposons are discrete DNA sequences capable of *rec* A independent transposition (14); they cannot replicate autonomously but must be integrated in a replicon (14). Many of them control resistance to antibiotics, and some of them govern the metabolism of certain substrates (9,12).

In a review paper, Kleckner et al (14) summarized transposon properties and hypothesized their potential value as genetic tools; ie, as mutagenic agents, a means of obtaining deletions, or homology between replicons. In addition, in the case of bacteria associated with plants, transposons allow "tagging" of genes governing the relationship with plants (4).

Insertion of bacteriophage Mu genome in a P-1 incompatibility group plasmid dramatically reduces the stable establishment of the plasmid in transconjugant bacteria (21). Due to this "suicide effect," transposons have been easily introduced and used as mutagens in *Agrobacterium* (22) and *Rhizobium* (3). As in other gram-negative bacteria, P-1 group plasmids can be transferred into most strains of phytopathogenic *Pseudomonas* spp. (15,17). In *P. solanacearum* strain Kourou, it has been shown that RP4::Mu had no suicidal effect (5).

In this paper we report that P-1 group plasmids cannot stably establish themselves in *P. solanacearum* strain K60, but mutants of this strain have been isolated in which these plasmids can be transferred and stably maintained.

The inability of the wild type strain to maintain P-1 group plasmids was used to devise a method for introducing transposons Tn5, Tn7, and Tn10 into *P. solanacearum*. In this species transposons can hop and insert into the bacterial genome as shown by their mutagenic effects.

MATERIALS AND METHODS

Biological material. Table 1 lists the bacteria, phages, plasmids, and transposons used in this investigation.

Culture media and growth conditions. The complete peptone glucose (BG) and tetrazolium (BGT) media have been previously described (5). Minimal medium (MM) and peptone broth (B) were prepared according to Boucher and Sequeira (6). Unless otherwise stated, 10-ml broth cultures were grown at 32 C on a reciprocal shaker. When required, antibiotics were used at the following concentrations: tetracycline (Tc) 10 $\mu g \cdot ml^{-1}$; kanamycin (Km) 50 $\mu g \cdot ml^{-1}$; gentamycin (Gm) 40 $\mu g \cdot ml^{-1}$; nalidixic acid (Nal) 50 $\mu g \cdot ml^{-1}$; trimethoprim (Tp) 10 $\mu g \cdot ml^{-1}$; rifampicin (Rif) 50 $\mu g \cdot ml^{-1}$. Streptomycin (Sm) at 50 $\mu g \cdot ml^{-1}$ was used for Tn7-encoded resistance, whereas 100 $\mu g \cdot ml^{-1}$ was used to select chromosomal resistant mutants.

Conjugation. Matings were performed between exponentially growing strains in B broth (unless otherwise stated). A suspension containing 10^8 donor cells per milliliter was mixed with another containing 5×10^8 recipient cells per milliliter and collected by filtration on a Millipore HAWP filter (pore size 0.45 μm). Filters were incubated at 32 C on BG plates for the specified period of time, and the cells were resuspended in 5 ml of distilled water. Serial dilutions were plated on selective media. Controls consisted of each of the two parental strains incubated separately under the same conditions.

Plasmid extraction and agarose gel electrophoresis. Extraction of plasmid DNA and agarose gel electrophoresis were performed according to Casse et al (7). Electrophoresis was done in 0.7% agarose gel in tris-borate buffer at 4 $V \cdot cm^{-1}$.

RESULTS

Introduction of RP4 into strain K60. When strain Kourou, carrying the RP4 plasmid GM11000 (RP4), was mated overnight with a K60 derivative, CB9, no transconjugants occurred on BGT agar supplemented with Km and Sm. However, when recipient cells were previously grown (from an inoculum of 10^5 cells per milliliter) in B broth supplemented with acridine orange (AO) (75 $\mu g \cdot ml^{-1}$) or sodium dodecyl sulfate (SDS) (500 $\mu g \cdot ml^{-1}$) and washed in distilled water, transconjugants occurred with a frequency of 5×10^{-7} relative to the final number of donors. Similar results were obtained when the recipient cells were allowed to segregate for 6 hr following a 15-min mutagenesis with nitrosoguanidine (100 $\mu g \cdot ml^{-1}$) according to Miller (18). In all cases, transconjugants retained the unselected markers of the recipient (rifampicin

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resistance and auxotrophy). In addition, by cross-streaking on BGT agar, they were shown to be sensitive to phages PRR1 and PRD1 specific for cells harboring the plasmid RP4. Finally the presence of the RP4 plasmid was demonstrated by electrophoresis of DNA extracted from one of the CB9 (RP4) transconjugants (designated CB10) obtained after AO treatment (Fig. 1, lane B).

To determine if the RP4 transconjugants resulted from selection of genetically altered recipient bacteria, a naturally cured strain of CB10 was selected following growth in the presence of a mixture of 10^8–10^9 plaque-forming units of phages PRR1 and PRD1 and recovery of a kanamycin- and tetracycline-sensitive clone. This clone, designated CB18, was shown by agarose gel electrophoresis to be cured of RP4 (Fig. 1, lane C). This clone was mated for 3 hr with strain CB37 harboring either RP4 or pJB4J1. Selection was made on BGT + Sm + Km or BGT + Sm + Gm. Plasmid transfer occurred with a frequency of 5×10^{-4}. Plasmid transfer was not, however, detected when CB37 was crossed with CB9.

For convenience, the phenotype associated with the genetic alteration of acceptor bacteria will be called Nac$^-$, the wild type phenotype being Nac$^+$ (for nonacceptor).

Introduction of transposons in strain K60. If the inability of the wild type strain to acquire P-1 group plasmids was due to its inability to maintain rather than accept the plasmids, these P-1 group plasmids could be potential vectors for introducing transposons into *P. solanacearum*. The only way for transposons to be maintained in the recipients would be to transpose into another replicon originally present in the bacterium. Theoretically, any donor bacterium could be used in such experiments, but (for several reasons) we chose strain CB112 (a Nac$^-$ derivative of K60) in which the vector plasmids were first introduced. The use of nearly isogenic strains allowed us to work in culture conditions that were optimal for both donors and recipients and to avoid restriction phenomena and antagonisms that may have occurred if unrelated strains had been used.

Table 2 presents the matings, selections, and frequency of recipient bacteria that acquired the transposon-mediated antibiotic resistance. In all cases, no colonies were detected in the controls in which each parent strain was treated separately under similar conditions. These results show that a 2-hr mating (roughly one generation time) allows stable introduction of Tn5 and Tn10 at a frequency of 5×10^{-6} per donor cell. In contrast, transposition of Tn7 could be detected only following a 24-hr mating. Transposition of the Tn1 originally present on RP4 could not be checked due to the relatively high natural resistance of strain K60 to ampicillin.

At least 25 clones from each mating were restreaked twice on selective media and single colonies scored for the vector plasmid-encoded resistance to kanamycin (in the case of RP4:: Tn7::Tn10) or to gentamycin (in the case of pJB4J1). None of the clones tested had acquired these resistances.

Furthermore, agarose gel electrophoresis performed on DNA extracted from five clones harboring Tn5 or Tn7 did not reveal the presence of covalently closed circular DNA (CCC DNA) (Fig. 2,

TABLE 1. Transposons, phages, plasmids, and bacterial strains

Strains	Relevant characters	Origin or reference
Pseudomonas solanacearum		
Strain Kourou		
GM1 1000	Wild type	(17)
Strain K60 and derivatives		
K60	Wild type	(6)
CB9	*met-3, trp-1, rif-3, str-3*	(6)
CB10	*met-3, trp-1, rif-3, str-3* Nac$^-$ (RP4)[a]	This paper
CB18	*met-3, trp-1, rif-3, str-3* Nac$^-$	CB10 cured of RP4
CB37	Nac$^-$	AO[b] treatment of K60
CB106	*str-3, rif-5, cyc-1, nal-1, spc-1*	Our collection
CB112	*met-4, gly-1, str-14,* Nac$^-$	Our collection
CB177	*nal-17*	Our collection
Plasmids		
RP4	Ap, Tc, Km	(21)
pPH151	Gm	(3)
pJB4J1	Gm, Tn5 lysogenic for Mu	(3)
RP4::Tn7	Tn7 inserted in Tc determinant	Panopoulous
RP4::Tn7::Tn10	Integration of Tn10 in RP4::Tn7	Our collection
Phages		
PRR1	) specific for bacteria harboring P-1 group	(20)
PRD1	) plasmid	(19)
Transposons		
Tn5	Km	(2)
Tn7	Sm, Sp, Tp	(1)
Tn10	Tc	(13)

[a] Nac$^-$: ability to stably maintain P-1 incompatibility group plasmid (see text).
[b] AO, acridine orange.

TABLE 2. Frequencies of recipients harboring transposons Tn5, Tn7, or Tn10 vs final number of donor cells following conjugation of a Nac$^+$ strain with a donor harboring the transposon inserted in a P-1 plasmid

Nac$^-$ donor→	CB112 (RP4::Tn7::Tn10)		CB112 (pJB 4J1)
Nac$^+$ recipient→	CB177		CB106
Selection plate→	BGT + Sm + Nal	BGT + Tc + Nal	BGT + Km + Nal
Transposon studied→	Tn7	Tn10	Tn5
Frequencies			
2 hr mating→	$<10^{-8}$	5×10^{-6}	5×10^{-6}
24-hr mating→	5×10^{-8}	...[a]	10^{-6}

[a] ..., Not tested.

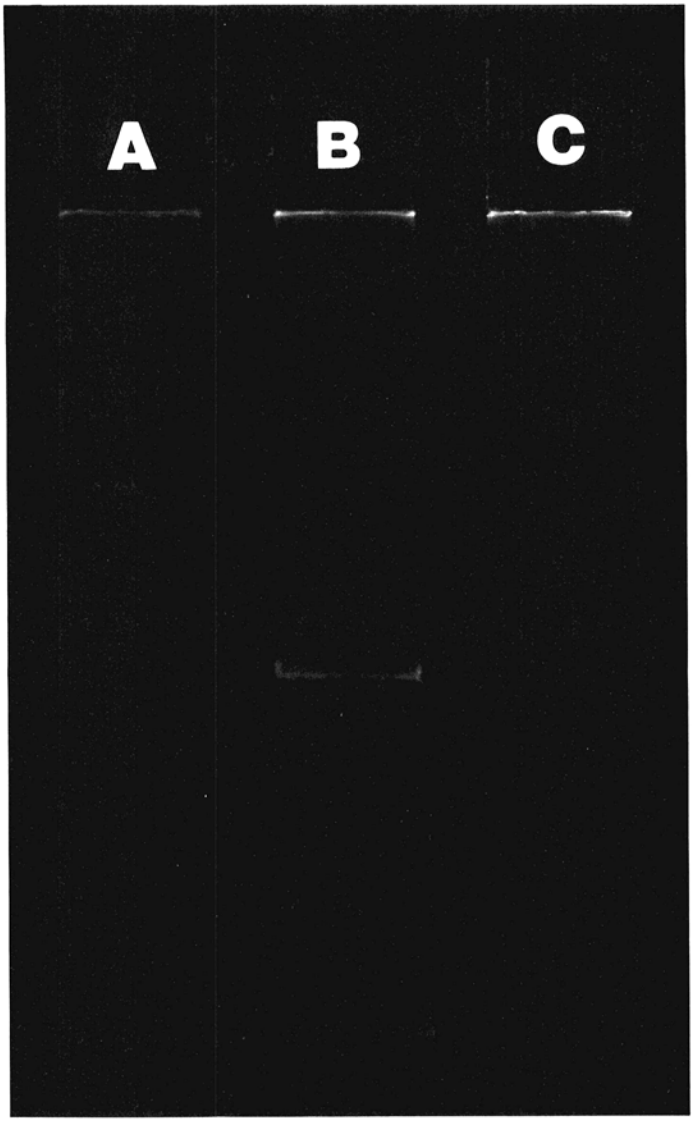

Fig. 1. Agarose gel electrophoresis of plasmid DNA from crude lysates of *Pseudomonas solanacearum* strains: A, CB9; B, CB10; and C, CB18.

lanes B–K).

K60::Tn5 derivatives were obtained following conjugation between K60 and CB112 (pJB4J1). Plasmid pPH151 was introduced into one of them following growth in the presence of SDS. When K60::Tn5 (pPH151) obtained that way was mated for 6 hr with CB18 followed by selection on BGT + Sm + Rif + Kana, transconjugants harboring the Tn5-encoded resistance occurred with a frequency of 10^{-7}. These transconjugants were shown to have received a pPH151::Tn5 hybrid plasmid since they always cotransferred kanamycin and gentamycin resistances. This further demonstrates the occurrence of transposition in *P. solanacearum*.

Transposon-induced auxotrophic mutations. Since insertion of a transposon into a gene can have a mutagenic effect (14), we looked for the presence of auxotrophic mutants among independent CB106::Tn5 and CB117::Tn10 clones. Of 763 Tn5-harboring clones tested, seven auxotrophs were detected. In a nonmutagenized population, the frequency of spontaneous auxotrophic mutants was several orders of magnitude lower. Four of the mutants obtained differed in amino acid requirements (aromatic amino acids, serine, aspartate, and cysteine). The nutritional defect could not be determined for the other three. Five of the mutants did not exhibit detectable reversion (apparent frequency $< 10^{-10}$), whereas two mutants reverted to prototrophy with an apparent frequency of 10^{-9}. These reversions were always associated with a loss of kanamycin resistance and, therefore, probably resulted from excision of the transposon.

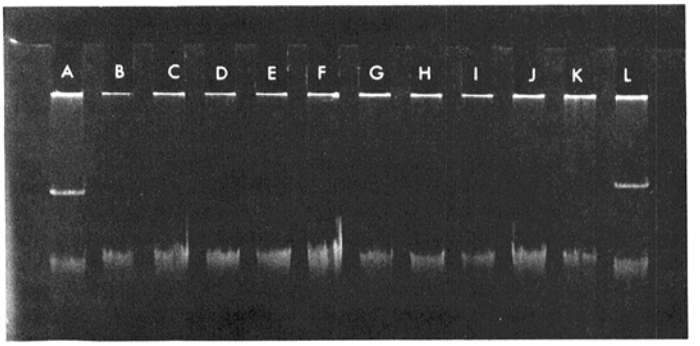

Fig. 2. Agarose gel electrophoresis of crude lysates from five independent clones of *Pseudomonas solanacearum* CB106::Tn5 (lanes B–F) and CB177::Tn10 (lanes G–K). In the same experiment strains CB10 (lane A) and CB37 (RP4::Tn7) (lane L) were included as controls.

Of the 766 Tn10-harboring clones tested, eight were found to be auxotrophic.

DISCUSSION

We have shown that by using P-1 group plasmids, transposons were introduced into Nac^+ K60 strain. This demonstrates that these plasmids were transferred into these bacteria. Since no CCC DNA was found in the transposon-harboring clones, it is concluded that the vector plasmid is not maintained in Nac^+ bacteria, and that the only way for the transposons to be maintained is by transposition into the genome of the recipient cell.

One possible hypothesis for the inability of P-1 group plasmids to be maintained in strain K60 could be the presence of a plasmid of the same incompatibility group. That growth in the presence of AO or SDS (two substances known to be curing agents [10,11]) allows the isolation of K60 derivatives that can accept two P-1 plasmids, is in agreement with this hypothesis. However, by using an extraction procedure known to be efficient for the isolation of plasmids of molecular weight up to 300 M daltons (7,8), it was not possible to demonstrate the presence of a plasmid in the wild type strain (Fig. 1, lane A). In addition, strain CB9 does not allow propagation of phages PRR1 or PRD1 (*unpublished*), which are known to be specific for strains harboring P-1 group plasmids. It should be noted that, in contrast to what has been found in the case *P. solanacearum* strain Kourou (16), derivatives of K60 obtained after growth in the presence of AO had retained their pathogenic properties (*unpublished*).

Owing to the inability of strain K60 to stably maintain P-1 plasmids, it was possible to mutagenize its cells with transposons. This finding should greatly facilitate a genetic study of the bacterial functions involved in pathogenesis. Tn5 and Tn10 should be particularly helpful since they allow recovery of many independent mutants in a single experiment; both mutagenesis and selection occurs on agar media during a single generation time.

By using a transposon that integrates at random we can determine the frequency of avirulent mutants among bacteria that have acquired a transposon. This should permit estimation of the number of genes that affect virulence.

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