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SHORT COMMUNICATIONS

A Vehicle for the Introduction of Transposons into Plant-Associated Pseudomonads

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A recombinant plasmid with wide-host-range transfer functions, narrow-host-range replication functions, and carrying a kanamycin-resistant transposon transferred kanamycin resistance to a number of plant-associated pseudomonads. Southern hybridization studies suggest that only a small portion of the plasmid, coinciding with the location of the transposon, is present in the kanamycin-resistant *Pseudomonas* derivatives. The plasmid sequences appear to be inserted at a number of different sites in the recipient genome. This plasmid can thus be used as a vehicle for the introduction of transposons into some plant-associated pseudomonads and should be useful in both genetic and ecological studies of these bacteria.

The plant-associated pseudomonads are a diverse and agriculturally important group of bacteria. They include phytopathogens as well as saprophytes, and recent experiments have suggested the potential of using some of these bacteria as biological agents for plant growth promotion (*14*) and disease control (*8,13,17*). As a group, these bacteria produce a wide variety of secondary metabolites, including phytotoxins, siderophores, and antibiotics (*5*). Many studies involving plant-associated pseudomonads can benefit from a genetic approach, which has played an instrumental role in molecular studies of *Escherichia coli* and related bacteria. Nevertheless, this approach has rarely been used, largely because of the lack of suitable tools. In this report we describe a genetic tool for carrying out transposon (*15*) mutagenesis in plant-associated pseudomonads.

To obtain transposon mutagenesis, one needs a vehicle which can introduce the drug-resistant transposon into the target cells but which cannot be maintained autonomously in these cells (*4*). Under such conditions, the transposon carried on the vehicle cannot be inherited, and only those bacteria which have the transposon inserted into their genome will become stably drug resistant. The recombinant plasmid pRK2013 (*2*) appeared to us to have all the required properties. This plasmid was constructed by Figurski and Helinski during their study on the replication of the broad-host-range plasmid RK2. It (Fig. 1A) is composed of an *Eco*RI fragment of the RK2 derivative pRK212.2 inserted into the unrelated plasmid ColE1. This *Eco*RI fragment contains the transfer functions of RK2 but only part of the replication apparatus. It cannot self-replicate and the replication of pRK2013 is dependent of the ColE1 replicon, which has a relatively narrow host range. A portion of the *Eco*RI fragment was derived from the recombinant plasmid pML31 [which was ultimately derived from the R factor R6-5 (*6*)] and contains the kanamycin-resistant transposon Tn*903* (*9,3,10*). We describe below experiments which indicate the pRK2013 can be used to introduce Tn*903* into some plant-associated pseudomonads for the purpose of carrying out transposon mutagenesis in these bacteria.

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0147-619X/85 $3.00

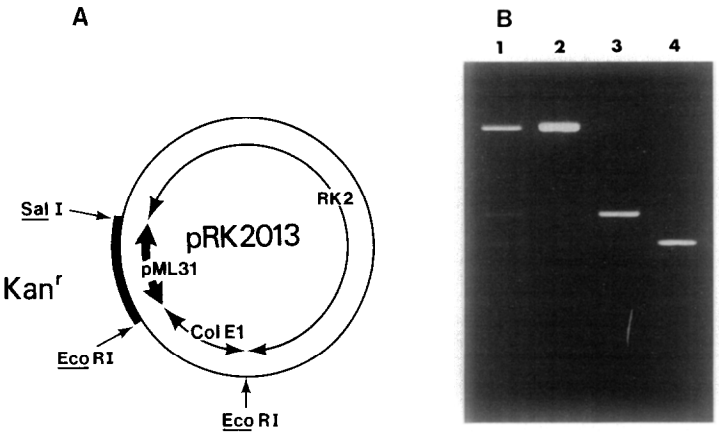

FIG. 1. (A) The composition of pRK2013, reconstructed from information in reference (*2*). (B) Agarose gel analysis of pRK2013 and the purified constituent fragments. Lane 1 contains pRK2013 digested with *Sal*I and *Eco*RI; lane 2, the RK2-derived fragment (ca. 36.7 kb); lane 3, the ColE1-derived fragment (ca. 6.3 kb); and lane 4, the pML31-derived fragment (ca. 5 kb).

A slightly different approach has been used by Sato *et al.* (*12*) to introduce Tn*7* into phytopathogenic *Pseudomonas*.

E.coli was grown in nutrient broth (Difco) containing 0.5% NaCl. For *Pseudomonas*, the defined minimal medium (PM) of Panopoulos *et al.* (*11*) and the rich medium *Pseudomonas* F agar (Difco) were used. For routine culturing of *Pseudomonas* strains in liquid, PM containing 0.1% proteose peptone No. 3 (Difco) was used. Kanamycin was used at a final concentration of 50 μg/ml. All *Pseudomonas* strains used in these studies and their sources are listed in Table 1.

Donor and recipient bacteria were grown separately to late log phase (approximately 5×10^8 cells/ml). An aliquot (0.2 ml) of a 1:1 mixture of the cultures was spotted onto a 25-mm Millipore filter placed atop nutrient agar medium. After overnight incubation at 28°C, the filter was washed with sterile water and appropriate dilutions were plated on selective medium (PM containing 50 μg/ml kanamycin) and nonselective medium (nutrient agar). As control, the donor and recipient parents were subjected, separately, to the same treatments.

Procedures for DNA isolation and Southern hybridization were as described in Davis *et al.* (*1*). Probe DNA was radioactively labeled ([^{32}P]dCMP), using a nick translation system from New England Nuclear Corporation. All restriction enzymes were obtained from New England Biolabs, Inc. and used according to the manufacturer's instructions.

Various *Pseudomonas* strains were used as recipients in bacterial conjugation experiments. The donors used were derivatives of the *E. coli* strain HB101 carrying either RK2 (from J. Jaynes) or pRK2013 (from D. Helinski). Selection was for the ability to grow on the minimal medium PM containing 50 μg/ml kanamycin. *E. coli* strain HB101 is multiply auxotrophic, and the *Pseudomonas* strains are kanamycin sensitive, thus the only survivors are *Pseudomonas* derivatives which either have mutated spontaneously to kanamycin-resistance (Kan^r), or have received the Kan^r determinant from the *E. coli* donor. Since spontaneous Kan^r mutants of the *Pseudomonas* strains occur at frequencies too low to be detected ($<10^{-9}$, see Table 1), except for the *P. solanacearum* strains (approximately 10^{-8}), the Kan^r derivatives we obtained (Table 1) are most likely transconjugants. The frequencies of Kan^r transconjugants range over several orders of magnitude in the various *Pseudomonas* strains, reflecting the genetic diversity among these strains. These differences could be at the step of

TABLE 1

Pseudomonas RECIPIENT STRAINS, THEIR SOURCES, AND THE FREQUENCIES OF KANAMYCIN-RESISTANT DERIVATIVES

Recipient	Source	No. of Kan^r derivatives/parental bacterium		
		Donor		
		RK2	pRK2013	No donor
Fluorescent *Pseudomonas*[a]				
MSU 4	D. Sands	3×10^{-1}	1×10^{-6}	$<10^{-9}$
MSU 20	D. Sands	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
MSU 37	D. Sands	7×10^{-6}	$<10^{-9}$	$<10^{-9}$
MSU 47	D. Sands	2×10^{-5}	4×10^{-7}	$<10^{-9}$
MSU 76	D. Sands	2×10^{-2}	4×10^{-7}	$<10^{-9}$
MSU 80	D. Sands	3×10^{-2}	6×10^{-6}	$<10^{-9}$
MSU 148	D. Sands	4×10^{-6}	1×10^{-8}	$<10^{-9}$
MSU 174	D. Sands	5×10^{-1}	2×10^{-6}	$<10^{-9}$
MSU 178	D. Sands	2×10^{-5}	$<10^{-9}$	$<10^{-9}$
MSU 188	D. Sands	1×10^{-2}	1×10^{-8}	$<10^{-9}$
MSU 193	D. Sands	7×10^{-2}	1×10^{-8}	$<10^{-9}$
P. syringae pv. *savastanoi*				
S2009	T. Kosuge	nt[b]	$<10^{-9}$	$<10^{-9}$
P. syringae pv. *phaseolicoli*				
HB33	S. Patil	nt	$<10^{-9}$	$<10^{-9}$
HB36	S. Patil	nt	$<10^{-9}$	$<10^{-9}$
P. solanacearum				
K60	A. Kelman	1×10^{-8}	8×10^{-9}	1×10^{-8}
S207	A. Kelman	5×10^{-6}	1×10^{-8}	9×10^{-9}

[a] These strains were isolated from plant material. Taxonomically, they belong to the *P. syringae* and *P. fluorescens-putida* groups (B. Hemming, personal communication).

[b] Not tested.

plasmid transfer, e.g., some strains may not be compatible with the RK2 transfer apparatus, or they could be at subsequent steps, including DNA restriction systems and plasmid maintenance in the recipients.

All the strains which yielded Kan^r transconjugants with the pRK2013 donor also yielded Kan^r transconjugants with the RK2 donor, and at much higher frequencies. These results are consistent with the possibility that pRK2013 (ColE1 replicon) cannot be maintained in the *Pseudomonas* recipients, and that the Kan^r derivatives we observed are the consequence of transposition of the Kan^r transposon Tn*903* carried on pRK2013. This hypothesis is supported by the observations that plasmids of donor size could be detected in the RK2-derived Kan^r *Pseudomonas,* and that these strains could transfer the Kan^r phenotype back into *E. coli,* whereas the results were negative on both counts for the pRK2013-derived Kan^r *Pseudomonas* (data not shown). In addition, we describe below Southern hybridization studies with one of the strains (MSU174) and its pRK2013 derived Kan^r transconjugants which confirm that only a small portion of pRK2013, coinciding with the location of Tn*903,* is present in the Kan^r derivatives.

The plasmid pRK2013 has two *Eco*RI sites (Fig. 1A) and, as expected, two hybridized fragments were observed with the donor parent strain HB101 (pRK2013) (Fig. 2, lane 1). Strain HB101 showed no hybridization

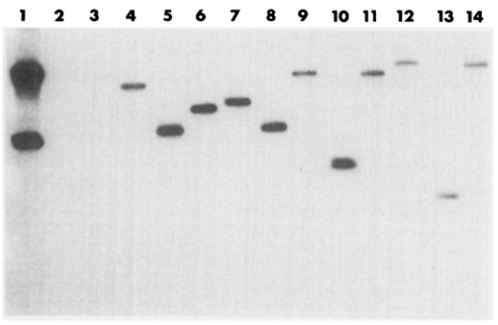

FIG. 2. The Kan^r *Pseudomonas* derivatives have received and retained pRK2013 sequences. Autoradiograms of Southern blot containing DNA from the following strains: lane 1, the donor parent HB101 (pRK2013); lane 2, HB101; lane 3, the recipient parent MSU174; lanes 4–14, MSU174 Kan^r derivatives. The DNA was digested with *Eco*RI; the probe used was pRK2013.

(Fig. 2, lane 2), confirming that what we observed were indeed pRK2013 sequences. The recipient parent strain MSU174 also showed no hybridization (Fig. 2, lane 3), indicating that it contained no DNA sequence homologous to pRK2013. Each of the MSU174 Kan^r derivatives examined (11 shown here, Fig. 2, lanes 4–14), chosen at random, showed a different hybridization pattern and each was different from that of HB101 (pRK2013), suggesting that the plasmid has undergone structural changes in these strains. These hybridization patterns may be the results of TN*903* transpositions. Alternatively, pRK2013 may contain, near one of its *Eco*RI sites, function(s) which adversely affect the stable maintenance of the plasmid in *Pseudomonas*. Selection for stable Kan^r would then yield independent deletion derivatives of pRK2013, most of which would have lost one *Eco*RI site.

To differentiate between the above possibilities, the extent of pRK2013 sequences retained in the Kan^r transconjugants was examined. The plasmid was digested with *Sal*I and *Eco*RI, yielding three fragments: a ca. 36.7-kb fragment derived from RK2, a ca. 6.3-kb fragment derived from ColE1; and a ca. 5-kb fragment (which contains Tn*903*)

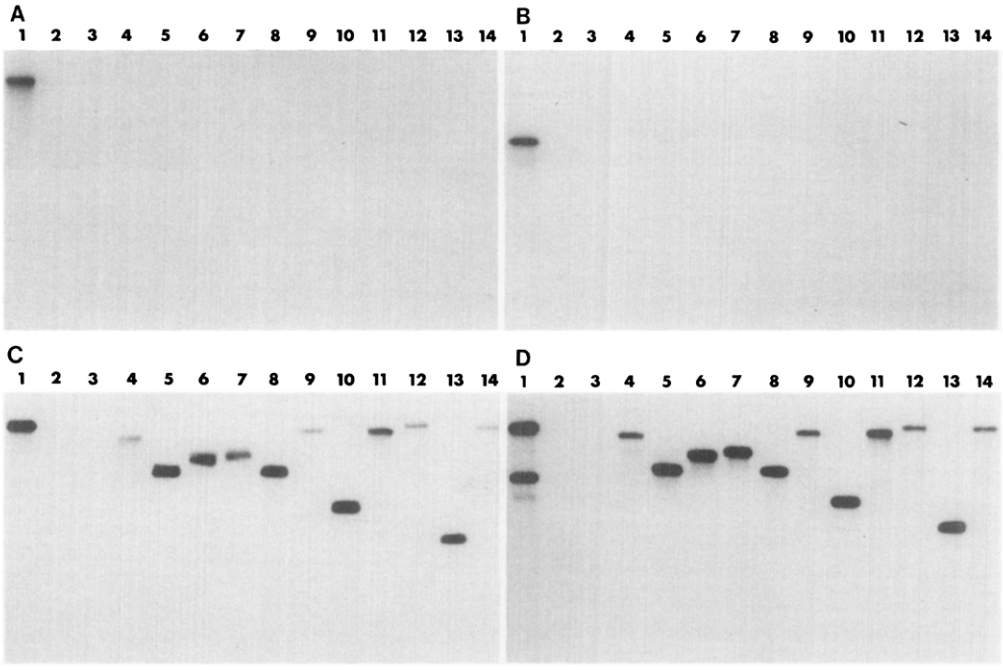

FIG. 3. Only a small portion of pRK2013, coinciding with the location of Tn*903*, is present in the MSU174 Kan^r derivatives. The experimental procedure and the arrangement of bacterial strains were identical to those indicated in Fig. 2. The probes used were: (A) the RK2-derived fragment; (B) the ColE1-derived fragment; (C) and (D) after a gentle rinse with hybridization buffer, A and B were reprobed with the ca. 5-kb fragment (derived from pML31) to yield C and D, respectively.

derived from pML31 (Fig. 1A). The fragments were separated by preparatory agarose gel electrophoresis and recovered by electroelution [(*7*), Fig. 1B]. When the RK2-derived fragment and the ColE1-derived fragment were used as probes in separate experiments, no hybridization was observed between these fragments and the Kan^r transconjugants (Fig. 3A and B) indicating that these portions of the plasmids were not retained in the transconjugants. When these transconjugants were reprobed with the pML31-derived fragment, hybridization patterns identical to those obtained with the entire plasmid were observed (Fig. 3C and D).

We conclude that MSU174 exconjugants which have become kanamycin resistant have retained a small portion of the donor plasmid pRK2013. This portion is contained entirely within the 5-kb pML31-derived fragment, 3.1 kb of which is the kanamycin-resistant transposon Tn*903*. We believe that these Kan^r transconjugants are indeed the results of transposition of Tn*903* into the recipient genome.

The hybridized fragments in the transconjugants vary in size from less than 6 kb to more than 30 kb. Since the inserted DNA is less than 5 kb, this size variation must reflect the insertion of plasmid DNA into different sites in the recipient genome. In addition, only one hybridized fragment has been observed in each of the Kan^r transconjugants we have examined so far (>70), indicating that only one transposition event has occurred in each of these strains. The attributes described above suggest that the pRK2013/Tn*903* system should be useful for transposon mutagenesis in plant-associated *Pseudomonas* species.

Strain MSU174 produces an antimycotic which has been shown to inhibit the growth of *Ceratocystis ulmi*, the causative agent of Dutch elm disease and *C. montia*, the blue stain fungus of pine. This *Pseudomonas* is being tested as a potential agent for controlling these diseases. To determine the role of antimycotic production in disease control, we have used pRK2013 to isolate a large number of insertion mutants which are defective in antimycotic production (manuscript in preparation). Linkage between insertion and mutant phenotype has been shown in the mutants examined.

Recently, Simon *et al.* (*16*) have constructed a versatile system of plasmids based on the same considerations (*4*). Although the plasmids have been tested mainly on *Rhizobium*, given what we now know, they should also be useful for at least some plant-associated *Pseudomonas* spp.

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