
Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of *Agrobacterium tumefaciens*

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ABSTRACT

A procedure is presented, that has allowed the rapid assignment of transposon Tn1 and Tn7 insertion sites in the large (130 Md) nopaline Ti-plasmid pTiC58, to specific restriction enzyme fragments.

Total bacterial DNA is isolated from *Agrobacterium tumefaciens* strain C58 mutants that carry a transposon in their Ti-plasmid, and digested with an appropriate restriction endonuclease. The fragments are separated on an agarose gel, denatured and transferred to nitrocellulose filters. These are hybridized against purified wild type pTiC58, or against segments of pTiC58, cloned in *E. coli* using pBR322 as a vector plasmid. DNA sequences homologous to the probe are detected by autoradiography, thus generating a restriction enzyme pattern of the plasmid from a digest of total bacterial DNA. Mutant fragments can be readily identified by their different position compared to a wild type reference.

This protocol eliminates the need to separate the large plasmid from chromosomal DNA for every mutant. In principle, it can be applied to the restriction enzyme analysis of insertion or deletion mutants in any plasmid that has no extensive homology with the chromosome.

INTRODUCTION

Mutagenesis by transposon insertion has proven to be very useful for studying the functional organization of plasmids (1-4).

Large plasmids, such as the tumour inducing (Ti) plasmids of *Agrobacterium tumefaciens* (5-7), must contain many different sites into which transposon insertion can occur, thereby eventually giving rise to alteration of a known plasmid phenotype (8).

The location of transposon insertion sites on a plasmid map is usually achieved by isolating plasmid DNA from the mutant strain, followed either by analysis of its restriction enzyme digest pattern, or electron microscopic studies of heteroduplex molecules.

However, preparative plasmid isolation, particularly in the case of the large Ti-plasmids, remains an expensive and time consuming step,

involving at least one CsCl gradient (9-10). Hence, the restriction enzyme analysis of a considerable number of mutant Ti-plasmids would be a formidable task if one had to separate plasmid from chromosomal DNA in each individual case.

Based on the technique of Southern (11) for detection of specific DNA sequences, we have developed a simple and rapid procedure for the simultaneous restriction analysis of many mutant plasmids, eliminating the requirement for plasmid purification from the mutant strains.

The method was applied to the mapping of Tn₁ and Tn₇ insertion sites in the nopaline Ti-plasmid of Agrobacterium tumefaciens strain C58 (pTiC58) and in the octopine Ti-plasmid of strain Ach5 (pTiAch5).

In this paper we present details of the procedure, along with some results obtained for Tn₁ insertions in pTiC58, which will illustrate its speed, reliability and wide range of possible applications.

MATERIALS AND METHODS

Bacterial Strains

Agrobacterium tumefaciens strain C58 was used as a source for purification of the nopaline Ti-plasmid pTiC58. Strain C58-C1 is a non-oncogenic derivative of C58, that has been cured of pTiC58 (13). E. coli strain C600 $r_K^- m_K^+$ was used to propagate recombinant plasmids containing segments of pTiC58 inserted in the HindIII site of pBR322 (12).

Buffers

TE buffer consists of 50 mM Tris pH 8.0, 20 mM EDTA. SSC contains 0.15 M NaCl, 0.015 M sodium citrate pH 7.0.

Mutagenesis of pTiC58 with Tn₁

A Tra^C pTiC58 derivative, pGV3100, was introduced by conjugation into A. tumefaciens strain C58-C1, already harbouring pGV5007, which is a Km^STra⁻ deletion mutant of the Tn₁ containing P-type plasmid RP4 (14). Selection was for transconjugants able to catabolize nopaline (Noc⁺). The isolation of pGV3100 mutants that had acquired Tn₁ from pGV5007 was done by conjugation, using as donor a Noc⁺ transconjugant C58-C1 (pGV5007) (pGV3100) and as acceptor a chloramphenicol resistant and erythromycin resistant (CIm^R Ery^R) derivative of strain C58-C1. Selection

was for carbenicillin resistant transconjugants. Subsequent tests for the various plasmid-borne phenotypes were performed as described elsewhere (15).

Isolation and restriction enzyme digestion of total bacterial DNA

Single colonies of mutant cells were grown at 28°C in 10 ml LB (16) to late logarithmic phase, harvested by centrifugation, and resuspended in 3 ml TE buffer. Sarkosyl was then added (1 ml of a 5 % solution in TE buffer), followed by pronase (1 ml of a 2.5 mg/ml solution in TE buffer). After incubation at 37°C for at least 1 h, the lysate was sheared by passing through a syringe without needle.

Subsequently, 1 ml of the sheared lysate was extracted twice with an equal volume of phenol, previously equilibrated with 0.5 M Tris, pH 8.0. The aqueous phase was extracted four times with ether. Finally, nucleic acids were precipitated from the aqueous phase at -20°C by the addition of two volumes 96 % ethanol. The pellet was redissolved in 0.1-0.2 ml of 5 mM Tris, pH 8.0, 5 mM NaCl, 0.5 mM EDTA.

Digestions with restriction endonucleases were usually carried out on 1-3 µg of total bacterial DNA, in a final reaction volume of 40 µl. The following enzymes were used, under conditions described elsewhere (12): HindIII, HpaI, SmaI, KpnI and BamHI.

Agarose gel electrophoresis and transfer of DNA to nitrocellulose filters

Digestion products were separated on (180 x 135 mm) horizontal 0.8 % agarose slab-gels, in 40 mM Tris-acetate buffer, pH 8.0, containing 0.3 µg/ml ethidium bromide. One half of the digest reaction mixture was loaded into a slot. After electrophoresis (25 mA, 18 h) the gel was photographed under ultraviolet light. Subsequently, DNA was denatured and neutralized within the gel as described by Southern (11). Transfer to nitrocellulose filters (Sartorius) was accomplished overnight in 20 x SSC, followed by heating of the filters at 80°C in vacuo for at least 2 h (11).

Preparation of ³²P-labelled plasmid DNA

Wild type pTiC58 was isolated as described (10) except that after collection from the first gradient the plasmid band was rerun for 48 h in a second CsCl-ethidium bromide gradient at 40,000 rpm in an R65 Beckman rotor.

Segments of pTiC58, resulting from partial HindIII digestion, were inserted in the HindIII site of pBR322, and introduced by transformation into E. coli C600 (12). The recombinant plasmids were isolated after CsCl-ethidium bromide equilibrium gradients of cleared lysates from the chloramphenicol-amplified E. coli cultures (17).

About 1 µg of plasmid DNA was ³²P-labelled by nick translation, essentially as described by Rigby *et al.* (18) in 0.1 ml final reaction mixture, using [α -³²P] ATP as the labelled precursor. After 4 h reaction, the unreacted triphosphates were removed by passing the mixture over Sephadex G-50.

Hybridization

The nitrocellulose filters, containing the denatured total bacterial DNA fragments, were pre-incubated at 68°C for at least 2 h in 3 x SSC, containing 0.02 % each of bovine serum albumin, ficoll and polyvinylpyrrolidone (19). For hybridization, this pre-incubation solution was supplemented with 0.5 % SDS and 1 mM EDTA.

³²P-labelled probe DNA (15-50 x 10⁶ cpm), denatured by boiling for 10 min, was added to the filters in a minimal volume of hybridization solution (usually 10-15 ml for 18 x 13cm filters). This was overlaid with paraffin and hybridization was carried out at 68°C overnight in closed boxes.

After hybridization, the filters were washed several times in 3 x SSC, 0.5 % SDS at 68°C, then twice in 3 x SSC. The sensitivity of autoradiography was enhanced using CaWO₄ intensifying screens (CAWO, West-Germany).

Enzymes and chemicals

Pronase, B grade, was from Calbiochem (San Diego, California). The restriction endonucleases HindIII and HpaI were purchased from New England Biolabs and SmaI was obtained from Boehringer (West-Germany).

[α -³²P] desoxyadenosine triphosphate (2000-3000 Ci/mmol) was from the Radiochemical Centre (Amersham, England). Sarkosyl was obtained from Ciba-Geigy (Basel, Switzerland).

RESULTS

Hybridization of total bacterial DNA with pTiC58

Digestion of total bacterial DNA from *A. tumefaciens* strain C58 with HindIII yields a complicated pattern of fragments after agarose gel electrophoresis (Fig. 1A). However, when these fragments are denatured, transferred to nitrocellulose filters, and hybridized with denatured pTiC58 DNA that has been purified and ³²P-labelled as described in Materials and Methods, only the homologous fragments, derived from the plasmid, are detected by autoradiography (Fig. 1B). This is demonstrated by the absence of clearly hybridizing fragments in the control track f, which contains a SmaI digest of total DNA from strain C58-C1, a derivative that has been cured from its Ti-plasmid. One thus generates the restriction enzyme pattern of pTiC58 from a digest of total bacterial DNA. The sensitivity is primarily dependent upon the specific activity of the radioactive probe. As shown in Figure 1, even 1 µg of total bacterial DNA, when hybridized against 3 × 10⁶ cpm/ml of ³²P-labelled pTiC58 produces a convenient pattern after 3 days autoradiography.

Large fragments, such as HindIII fragment 1, exhibit a variable, sometimes drastic, loss of intensity as compared to the ethidium bromide stained band in the agarose gel, probably due to incomplete transfer to the nitrocellulose filter. In later experiments, this has been partially overcome by exposing the upper part of the gel for 15 min to short-length UV irradiation.

In Fig. 2 and Fig. 3, the localization of transposon insertion sites in pTiC58 is illustrated. Total bacterial DNA was prepared from seven colony isolates, among which five had lost the capacity of inciting tumours on plants upon acquisition of Tn₁ by their Ti-plasmid. The DNA was digested separately with HpaI (Fig. 2) and HindIII (Fig. 3). Upon filter hybridization of the separated fragments with ³²P-labelled pTiC58 DNA, the HpaI and HindIII patterns of mutant plasmids can be readily compared to the wild type reference (Figs. 2 and 3, track d). Both HpaI and HindIII do not cleave Tn₁, so that the fragment in which insertion has occurred is shifted up from its normal position by 3.2 Md, but without intensity change, since the probe DNA is non-homologous with Tn₁.

For instance, the mutant plasmid in Fig. 2, track a, carries a Tn₁ in HpaI fragment 5. In track f, Tn₁ insertion has occurred in HpaI fragment 6. In tracks e

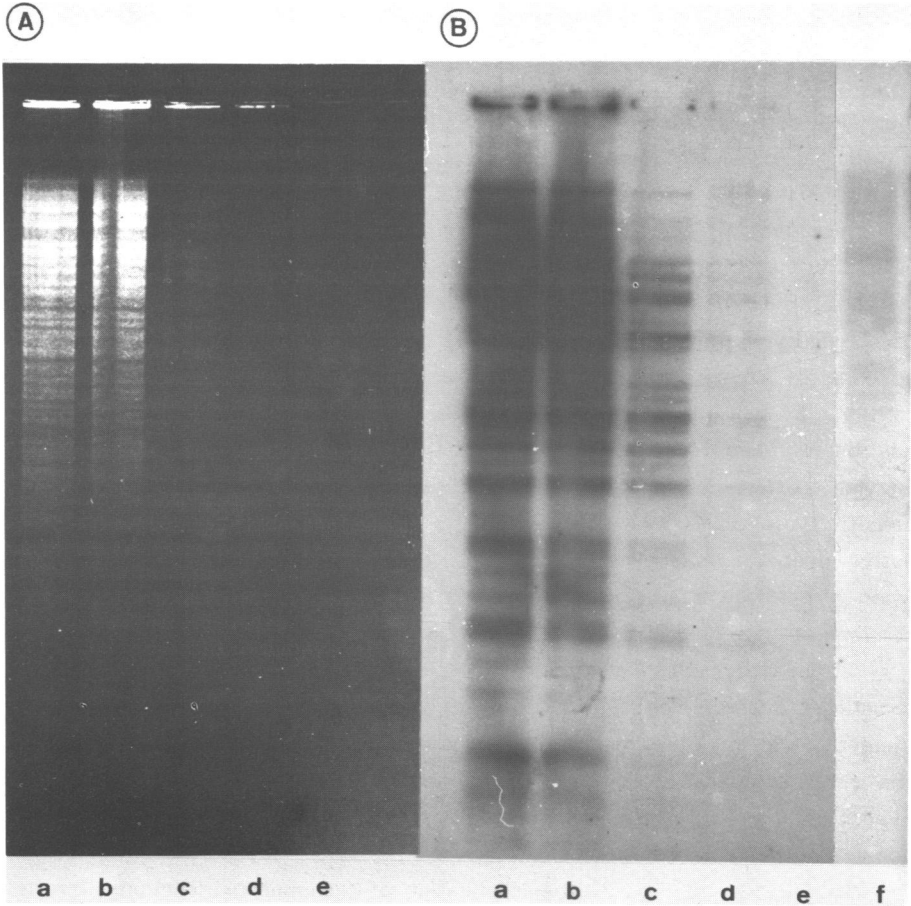


Figure 1. Hybridization of different amounts of HindIII digested total bacterial DNA from strain C58 with ^{32}P -pTIC58 (3×10^6 cpm/ml), prepared as described in Materials and Methods.

- A. Ethidium bromide-stained agarose gel-electrophoretic pattern. Tracks: a: 5 μg ; b: 3 μg ; c: 1 μg ; d: 0.1 μg ; e: 0.01 μg .
- B. Tracks a-e: pattern obtained after hybridization of the gel in Fig. 1A. Track f: hybridization of 5 μg SmaI digested total bacterial DNA from strain C58-C1 with ^{32}P -pTIC58. Exposure was for 3 days.

and g, HpaI fragment 2, normally present as a doublet with HpaI fragment 3 (see tracks a, b, c and f, since the wild type track d is overexposed in this area), is slightly shifted upward as a result of Tn1 insertion.

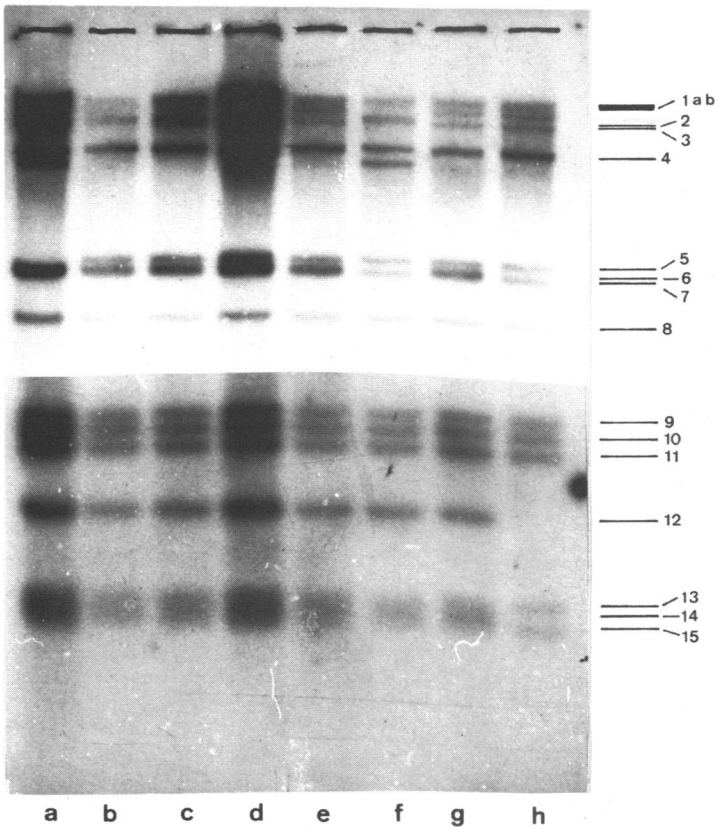


Figure 2. HpaI pattern of Tn1 insertion mutant pTiC58 plasmids. Gel electrophoresis, in 0.8 % agarose, of HpaI digests on 2 μ g of total bacterial DNA, prepared as described in Materials and Methods. Fragments were visualized after Southern transfer and hybridization of the total bacterial DNA fragments with 32 P-pTiC58 (3×10^6 cpm/ml). The upper part was exposed for 24 h, the lower part for 3 days.

Track a: mutant plasmid pGV3165; b: pGV3179; c: pGV3180; d: wild-type pTiC58; e: pGV3168; f: pGV3166; g: pGV3167; h: pGV3170.

Numbering of fragments produced by HpaI digestion of pTiC58 is indicated to the right.

Track h (Fig. 2) shows an interesting example of Tn1-promoted deletion formation: HpaI fragments 2, 6, 12 and 14 (contiguous on the restriction enzyme map - ref. 12) are missing, and the new fragment is found just above HpaI fragment 3. The deletion is calculated to involve a segment of 6.1 Md, normally part of the plasmid DNA region, which is transferred to the plant upon tumour induction (referred to as "T-DNA").

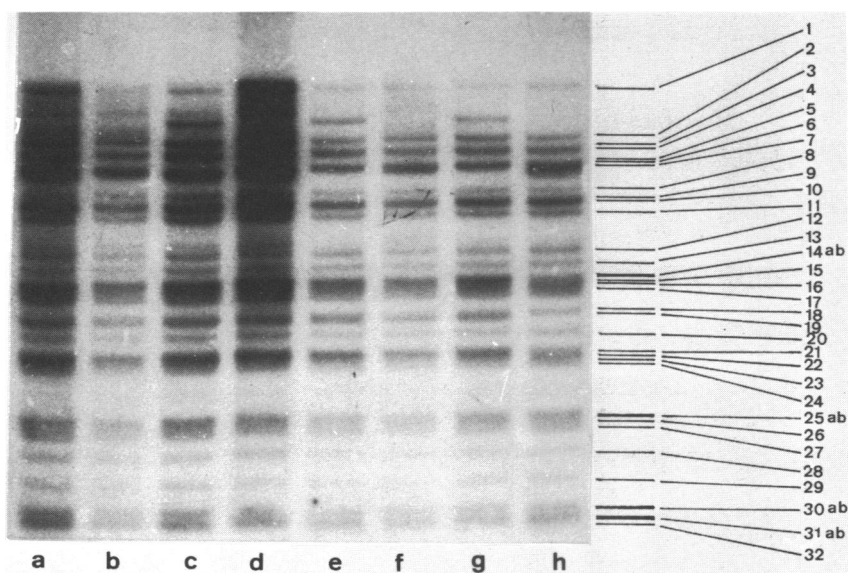


Figure 3. HindIII pattern of Tn₁ insertion mutant pTiC58 plasmids. Total bacterial DNA from the same mutants as in figure 2 was now digested with HindIII. Hybridization with ³²P-pTiC58 under the same conditions as in Figure 2. Exposure was for 24 h. Tracks a - h: see Figure 2. Numbering of fragments produced by HindIII digestion of pTiC58 is indicated to the right.

The large HpaI fragments 1a and 1b (48 and 29 Md respectively) are poorly resolved and incompletely transferred to the nitrocellulose filters. Tn₁ insertion in either of those fragments would not produce a detectable change in the banding pattern, as probably exemplified by the mutant plasmids in tracks b and c. The HindIII pattern of the same mutant plasmids, shown in Fig. 3, provides additional mapping information.

Insertions are readily localized in HindIII fragment 5 (track c) and fragment 7 (tracks e and g). This is consistent with the results obtained for those mutants by HpaI digestion, since HindIII fragment 5 partly overlaps HpaI fragment 1b, and a large part of HindIII fragment 7 is contained within HpaI fragment 2, as shown in the restriction map of pTiC58 (12).

The deletion mutant in track h lacks HindIII fragments 14, 19 and 22 of pTiC58.

Hybridization of total bacterial DNA with segments of pTiC58, cloned in pBR322

The HindIII digestion pattern of pTiC58 is still rather complex, with many fragments occurring as parts of doublet or even triplet bands (see Fig. 3). However recombinant plasmids have been constructed by insertion of DNA, resulting from partial HindIII cleavage of pTiC58, into the HindIII site of pBR322. The collection covers nearly all of the pTiC58 DNA sequence (12). Using such a recombinant plasmid as a ^{32}P -labelled probe in hybridization, the autoradiographic pattern obtained from a digest of total A. tumefaciens mutant DNA can be confined to those HindIII fragments of pTiC58, present in the probe. The Tn₁-containing fragment will always be detected, irrespective of its presence in the Ti-plasmid part of the probe, because of its homology with the Amp^R gene in pBR322.

In Fig. 4, ^{32}P -labelled pGV0342 DNA (Mw: 12.7 Md), containing the HindIII fragments 10, 14b and 15 of pTiC58, was hybridized against a filter with HindIII digested total bacterial DNA from three Tn₁ insertion mutants. Track c shows the wild type reference: a single band (HindIII fragment 10 from pTiC58) and a doublet (HindIII fragments 14b and 15) can be seen, in addition to one faint band, possibly of chromosomal origin, which does not interfere with unambiguous interpretation. Insertion has occurred in HindIII fragment 10 for the mutants in tracks a and d. Interestingly, mutant pGV3182 (track b) probably harbours two Tn₁ transposons in the same HindIII fragment (fragment 14b).

Fig. 5 shows the analysis of Tn₁ insertion mutants of pTiC58, defective in the catabolism of the unusual amino acid derivative nopaline. As ^{32}P -labelled probe, we used plasmid pGV0329 (16.9 Md), containing the HindIII fragments 2, 23, 26, 30, 31 and 33 of pTiC58. Track e shows the position of these fragments after digestion of DNA from the wild type C58 strain. Three Noc⁻ mutant isolates (tracks a, c and d) carry a Tn₁ inserted into the HindIII fragment 2, whereas in tracks b and f insertion has occurred into HindIII fragment 30, which is adjacent to fragment 2 on the restriction enzyme map of pTiC58 (see 12). Again one of the mutants (track c) possibly has acquired two Tn₁ transposons in HindIII fragment 2, since the mutant fragment is more than 3.2 Md longer than HindIII fragment 2.

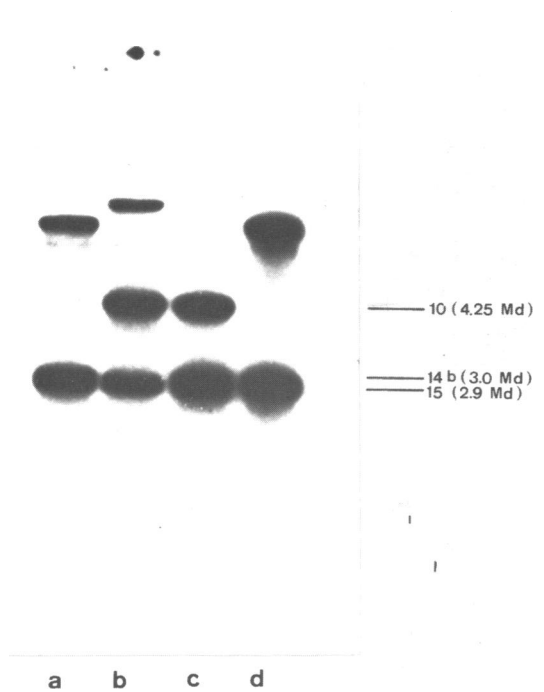


Figure 4. Hybridization of total bacterial DNA with ³²P-labelled pGV0342. Total bacterial DNA (1 µg) from three mutant strains was digested with HindIII. The fragments were separated electrophoretically (0.8 % agarose), denatured and transferred to a nitrocellulose filter. The recombinant plasmid pGV0342, containing the HindIII fragments 10, 14b and 15 of pTiC58 (contiguous on the physical map) inserted in the HindIII site of pBR322, was ³²P-labelled by nick-translation (1 µg was used), and served as a probe (5 x 10⁶ cpm/ml) for hybridization against the total DNA filter.
Track a: mutant plasmid pGV3181; b: pGV3182; c: wild-type pTiC58; d: pGV3183.

DISCUSSION

We made use of the DNA transfer and filter hybridization technique of Southern (11) to facilitate the restriction mapping of transposon insertion and deletion mutants in the large nopaline Ti-plasmid of Agrobacterium tumefaciens strain C58. This work has contributed to the establishment of a functional map for pTiC58 (15).

The major advantage of the procedure is that it avoids separation of

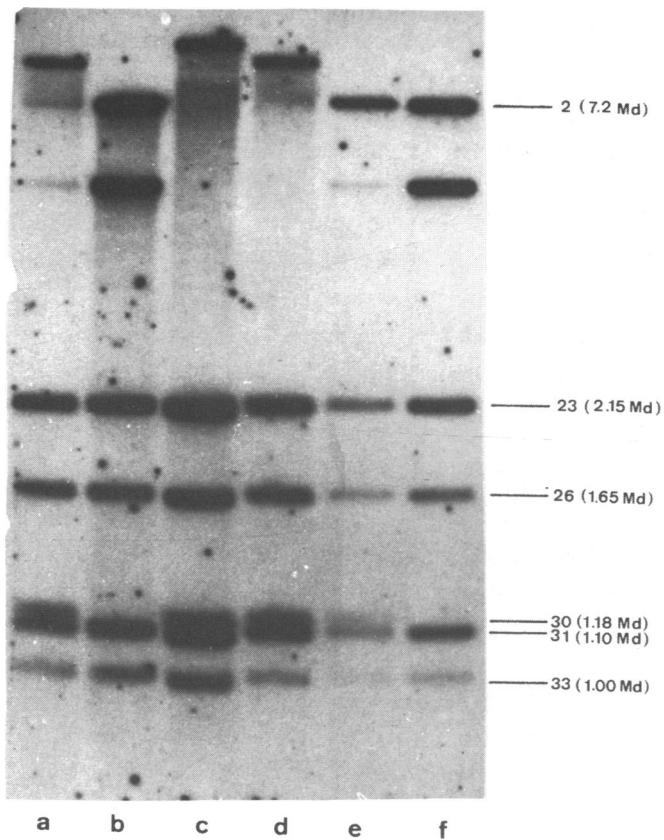


Figure 5. Hybridization of total bacterial DNA with ^{32}P -labelled pGV0329. Five mutant strains, unable to catabolize nopaline (Noc) were examined by hybridization of *Hind*III digested total DNA against ^{32}P -labelled pGV0329 (16.9 Md), containing the *Hind*III fragments 2, 23, 26, 30, 31 and 33 of pTiC58.

Track a: mutant plasmid pGV3184; b: pGV3172; c: pGV3175; d: pGV3173; e: wild-type pTiC58; f: pGV3176.

the large plasmid from chromosomal DNA. Plasmid isolation is required only to provide a radioactive probe for use in hybridization against total bacterial DNA, prepared from the mutant strains.

Isolation of total bacterial DNA is very simple and rapid, allowing the simultaneous restriction analysis of many mutants. Out of 2 ml late log phase bacterial culture, we isolate sufficient DNA for digestion with several different enzymes.

At this stage, however, we should emphasize that Agrobacterium tumefaciens in fact has been shown to harbour a second plasmid, which is even larger than the Ti-plasmid (20). Although the wild type C58 strain from which ^{32}P -labelled pTiC58 probe was prepared, also contains this additional plasmid (21), only Ti-plasmid sequences are detected after hybridization. Apparently, using our plasmid isolation procedure, the larger plasmid is selectively lost, so that our ^{32}P -labelled probe consists of virtually pure pTiC58 DNA. This is clearly shown by the absence of hybridizing fragments in total DNA from strain C58-C1, which is cured of its Ti-plasmid but still harbours the larger plasmid (see Results). The hybridization technique further permits selective analysis of specific plasmid segments. For that purpose, we made use of well-characterized recombinant plasmids, consisting of partial HindIII digestion products of pTiC58 inserted into pBR322, as ^{32}P -labelled probes. As shown in Figs. 4 and 5, the identification of transposon-containing fragments is greatly simplified when using these smaller probes.

It is clear that this protocol can be applied to the restriction enzyme analysis of any plasmid, and mutants derived from it, if there is no homology with the chromosome. Insertions or deletions are readily detected. However, it will be most advantageous in the restriction analysis of large plasmids that are difficult to purify, and especially when a large number of mutants is to be analyzed.

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