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Genetic Map of the Crown Gall Suppressive *Inc*W Plasmid pSa

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Summary. A genetic map of the W incompatibility group plasmid pSa has been prepared through the construction of deletion derivatives of pSa and the cloning of various fragments of pSa in pBR322. Phenotypic analysis of these derivatives has identified the location of genes encoding resistance to chloramphenicol, sulfonamides, spectinomycin, streptomycin, kanamycin, gentamycin, and tobramycin. Information sufficient for the replication of the plasmid in both *Escherichia coli* and *Agrobacterium tumefaciens* is contained within a 4 kilobase pair region. Two regions have been identified as involved in the transfer of the plasmid; one of these regions is also involved in the inhibition of oncogenesis by pSa when it is present in an oncogenic strain of *A. tumefaciens*. Certain of the deletion derivatives of pSa are potential vectors for the cloning and analysis of *A. tumefaciens* Ti plasmid DNA.

Introduction

The plasmid pSa belongs to the W incompatibility group. This group comprises conjugative plasmids of 30–35 kilobase pair (kbp) size in which a common homologous region of about 20 kbp contains genes involved in replication and transfer functions (Gorai et al. 1979). In addition to genes associated with plasmid replication and transfer, W group plasmids encode genes for resistance to a variety of antibiotics, for example, pSa confers resistance to the antibiotics chloramphenicol (Cm), kanamycin (Km), gentamycin (Gm), tobramycin (Tm), streptomycin (Sm), spectinomycin (Sp), and sulfonamides (Su) (Watanabe et al. 1968; Datta and Hedges 1971; Loper and Kado 1979). It has also been demonstrated that the presence of pSa in oncogenic strains of *Agrobacterium tumefaciens* interferes with the formation of crown gall tumors (Loper and Kado 1979; Farrand et al. 1981). Previous results suggested that pSa does not induce permanent genetic alterations in the Ti plasmid, which is present in oncogenic strains of *A. tumefaciens* and is known to be involved in oncogenicity. Determining how pSa inhibits crown gall oncogenesis may help in understanding the process of crown gall tumor formation.

In order to locate the regions of pSa involved in oncogenesis inhibition we have constructed a number of deletion derivatives of pSa and have cloned various regions of pSa on the plasmid pBR322 (Bolivar et al. 1977b). Analysis of the phenotypic properties of these pSa derivatives has allowed the construction of a genetic map of the plasmid including all the known phenotypes of pSa. Our results indicate that the inhibition of oncogenesis by pSa probably requires the function of genes involved in the mobilization and transfer of the R factor.

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Materials and Methods

Bacterial strains. Plasmids were constructed and characterized in the *E. coli* strains RR1 *pro*$^-$ *leu*$^-$ *B1*$^-$ *lacY Str*^{r} r_k^- m_k^- EndoI$^-$ (Bolivar et al. 1977a) and J53 *pro*$^-$ *met*$^-$ (Datta and Hedges 1971). *A. tumefaciens* strain NT1 *Rif*^{r} *Ery*^{r} (pTiC58 Tra^c) (J. Kao, K.L. Perry, and C.I. Kado, manuscript submitted) was constructed using strain NT1 (Watson et al. 1975). *E. coli* J53(pSa) was originally obtained from N. Panopoulos and J53 (pSa-1) from D. Bradley.

Media. Strains were routinely maintained on nutrient agar slopes or stabs. L-broth was used for liquid cultures and with 1.5% agar for solid medium (Miller 1972). For selection of *E. coli* strains, antibiotics were used at a concentration of 30 μg/ml Cm, 100 μg/ml Sm, 50 μg/ml Sp, 30 μg/ml Km, 5 μg/ml Gm, 10 μg/ml Tm, 30 μg/ml tetracycline (Tc), and 50 μg/ml ampicillin (Ap). Resistance to sulfonamides was determined using the method of Bauer et al. (1966). For selection of *A. tumefaciens* strains containing pSa derivatives, M-9 minimal medium containing 0.4% sucrose and 100 μg/ml Km was used.

Virulence Assays. Assays on carrot discs and *Kalanchoe* leaves were performed as described by Hamada and Farrand (1980). Assays on sunflower and tomato were performed according to Loper and Kado (1979).

Restriction Endonuclease Analysis. Enzymatic reactions were performed under conditions recommended by Bethesda Research Laboratories, Inc. (Rockville, Maryland). Agarose gels of 0.6–1.2% were used to analyze digested DNA (Bolivar et al. 1977a; Hamada et al. 1979).

Isolation of Plasmid DNA. Large quantities of plasmid DNA were purified as described by Bolivar et al. (1977a), Lin and Kado (1977), and Casse et al. (1979). Small amounts of plasmid DNA were prepared according to

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Kado and Liu (1981) for *A. tumefaciens,* and according to a procedure provided by B. Froman for *E. coli.* For the Froman miniscreen, 1 ml of overnight culture was centrifuged in a 1.5 ml tube for 5 min in an Eppendorf microfuge model 5412 and the supernatant discarded. The cell pellet was resuspended in 0.15 ml SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-HCl pH 8.0, 30 µg/ml pancreatic RNaseI) and lysed by the addition of 0.35 ml lytic mix [0.2 N NaOH, 1% Na dodecyl sulfate (SDS)]. Cells were lysed by 30 sec vigorous mixing, then the tubes were placed in an ice-water bath for 5 min to allow the SDS to precipitate. This was followed by the addition of 0.25 ml 3 M sodium acetate pH 4.2. After mixing, the tubes were returned to the ice-water bath for 15 min, then the precipitate removed by centrifugation for 10 min at 4° C in an Eppendorf microfuge model 5412. The supernatant was removed to a clean tube and an equal volume of cold isopropanol added. After several inversions to mix the solution, the precipitated DNA was collected by centrifugation for 10 min at *room temperature* in an Eppendorf microfuge. The resulting pellet was washed once with 70% EtOH at *room temperature*, and after removal of the ethanol wash, the pellet was dried for 15 min under vacuum and resuspended in 20 µl distilled H_2O. 1–2 µl of DNA was immediately digested with restriction enzymes and analyzed by gel electrophoresis.

Bacterial Conjugations. Intergeneric plasmid transfers were accomplished by mixing one colony of the *E. coli* donor with one colony of the *A. tumefaciens* recipient on an L agar plate. After 24–48 h growth at 29° C, cells from the surface of the plate were resuspended in 1 ml 50 mM Tris-HCl, pH 7.5. *A. tumefaciens* transconjugants were selected from the suspension on minimal medium containing Km.

Efficiency of plasmid transfer was determined with *E. coli* RR1 containing a pSa derivative as the donor and RR1 containing pBR322 as the recipient. Donor and recipient were mixed in L-broth at a ratio of 1:1 and allowed to incubate 15 h at 37° C. Potential donor, potential recipient, and transconjugant numbers were determined by plating dilutions on L-agar containing Km+Sp, Tc, and Km+Sp+Tc, respectively. Relative efficiency of transfer was expressed as:

$$\% = \frac{\dfrac{\#\text{ of transconjugants with pSa derivative}}{\#\text{ of available donors}}}{\dfrac{\#\text{ of transconjugants with pSa}}{\#\text{ of available donors}}} \times 10^2$$

Under these conditions, pSa was typically able to transfer to 20% of the available recipient cells.

Results

Construction of Derivatives of pSa. Restriction endonuclease cleavage of pSa DNA followed by agarose gel electrophoresis revealed the presence of one cleavage site for *Sal*I and *Sma*I, two sites for *Kpn*I and *Eco*RI, three sites for *Bam*HI, *Sst*II, *Bgl*II, and *Pst*I, and six sites for *Hin*dIII, as shown in Fig. 1. Successive digestion with one or more enzymes allowed the placement of these sites relative to one another to establish the linear restriction map of pSa (Fig. 2). Interestingly, 22 of 24 sites were in a 10 kbp region, with the remaining 19.6 kbp containing one cleavage site each for *Bgl*II and *Sst*II.

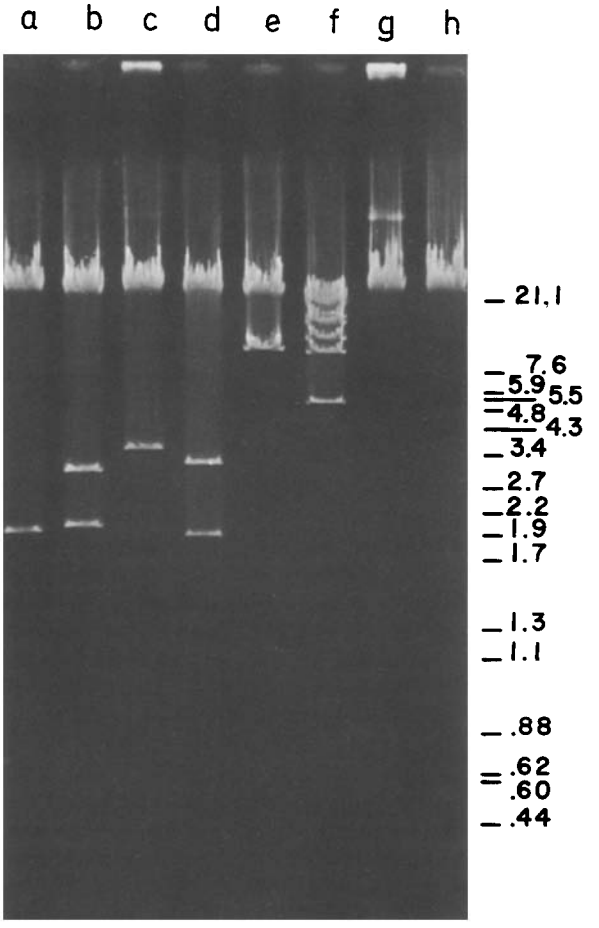

Fig. 1. Restriction endonuclease generated fragments of pSa DNA resolved by electrophoresis on a 0.8% agarose gel as described in Materials and Methods. Each lane was overloaded to make visible low molecular weight bands stained with ethidium bromide. pSa DNA was digested with: lane a) *Eco*RI; lane b) *Hin*dIII; lane c) *Kpn*I; lane d) *Pst*I; lane e) *Bam*HI; lane f) *Sst*II (partial digest); lane g) *Sal*I; lane h) *Sma*I

Based on the linear restriction map of pSa, a series of deletion derivatives of pSa were constructed by digesting pSa DNA with a restriction enzyme to release specific fragments of DNA, then ligating the digested DNA under conditions to favor circularization of the fragments. Bacteria were transformed with the ligated DNA and the transformed cells were plated on medium containing either Cm, Sm, Sp, or Km. Antibiotic resistant colonies were then tested for resistance to Cm, Km, Tm, Gm, Sm, Sp, and Su. Transformants with an altered antibiotic resistance pattern relative to pSa were assumed to be deletion derivatives of pSa.

The size and location of putative deletions were determined by purification and digestion of small amounts of DNA as described in Materials and Methods. The results of a *Bam*HI digestion of pSa and eight deletion derivatives are shown in Fig. 3. The altered electrophoretic pattern of the pSa derivatives is the result of the deletion of various regions of DNA. Successive digestion with various restriction enzymes (data not shown) established the location of the deletion in each of derivatives (Fig. 2). In all but one

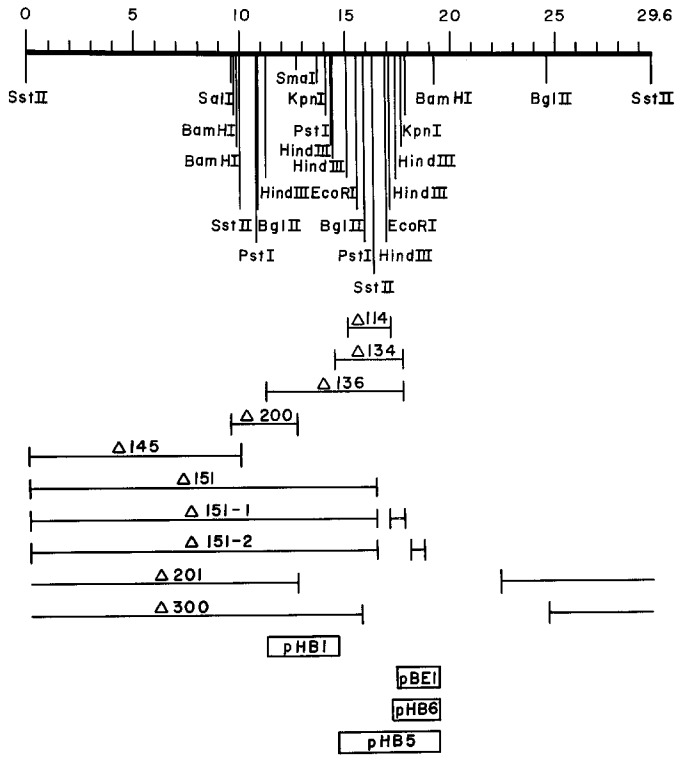

Fig. 2. Linear restriction map of pSa and its derivatives. One of the three *Sst*II sites has been designated as 0 kbp and the location of the other cleavage sites positioned accordingly. The scale in kbp is indicated across the top. The region deleted in each pSa derivative is indicated by a solid line below the map. For example, the region designated by Δ114 is deleted in pSa114. The open boxes indicate the regions cloned in the plasmid pBR322; the number in each indicates the recombinant in which this region is present

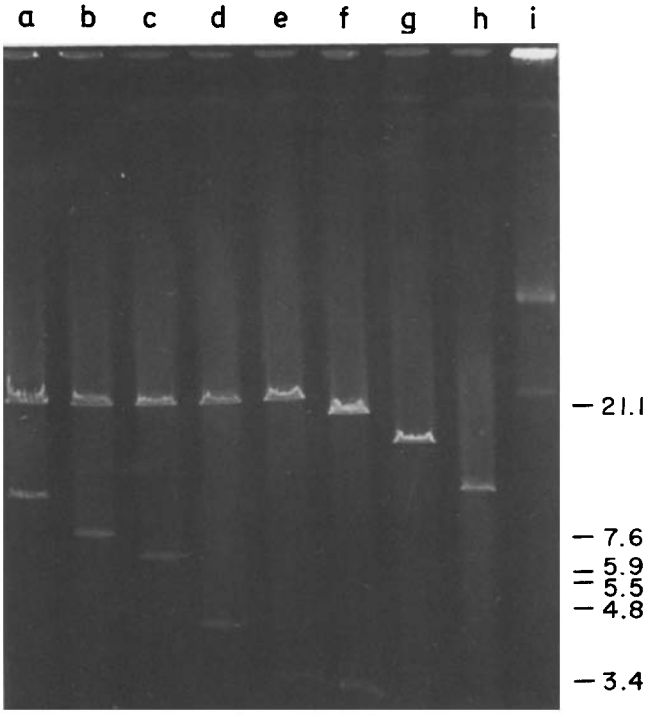

Fig. 3. Restriction analysis of deletion derivatives of pSa. Partially purified plasmid DNA from pSa derivatives was digested with *Bam*HI and subjected to agarose gel electrophoresis. Lane a) pSa; lane b) pSa114; lane c) pSa134; lane d) pSa136; lane e) pSa80; lane f) pSa200; lane g) pSa151; lane h) pSa201; and lane i) pSa300. Sizes are given in kpb

of the derivatives, the location of the deletion was consistent with the enzyme(s) used in the construction of the deletion. The exception is plasmid pSa201, which was obtained during the use of *Sal*I and *Sma*I to construct pSa200. The deletion in pSa201 apparently originates at the *Sma*I site but extends 14 kbp beyond the *Sal*I site, as illustrated in Fig. 2. Secondary deletions were introduced in the plasmid pSa151 to generate the plasmids pSa151-1 and pSa151-2. The construction of pSa151-1 was accomplished by the deletion of the two small *Hin*dIII fragments, while the construction of pSa151-2 involved the deletion of one of two small *Ava*II fragments present in pSa151 (Fig. 2).

In addition to constructing deletion derivatives of pSa, regions of pSa were cloned in plasmid pBR322. Recombinant Ap^r transformants were first identified by inactivation of the Tc^r gene due to insertions in the *Bam*HI or *Hin*dIII sites in the Tc gene on pBR322, then tested for each of the antibiotic resistances conferred by pSa. Recombinants containing pSa DNA were digested with the appropriate restriction enzymes to identify the cloned pSa DNA fragments. Four such recombinant plasmids are shown in Fig. 4, and the location of the cloned fragments on the pSa map is indicated in Fig. 2.

Phenotypic Characterization of pSa Derivatives. Four recombinant pBR322 plasmids containing pSa DNA were characterized with respect to their antibiotic resistance phenotype, and ten deletion derivatives of pSa were character-

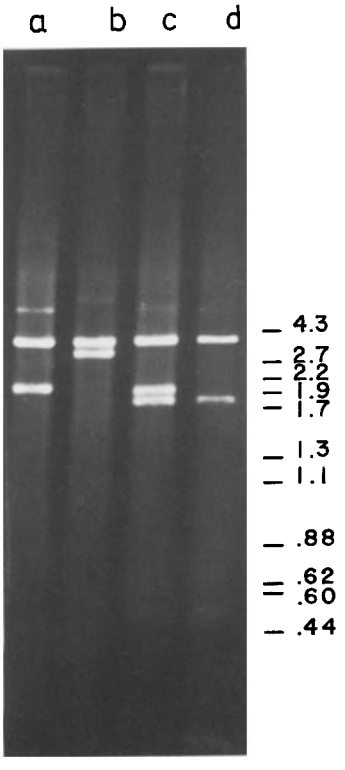

Fig. 4. Restriction analysis of recombinant derivatives of pSa. Partially purified plasmid DNA was digested with *Bam*HI and *Eco*RI (lane a) or *Bam*HI and *Hin*dIII (lanes b-d) and subjected to agarose gel electrophoresis. Lane a) pBE1; lane b) pHB1; lane c) pHB5; lande d) pHB6. Size are given kbp

Table 1. Phenotypic properties of pSa and its derivatives

Plasmid	Region deleted[a]	Antibiotic resistance[b]							% transfer	Oncogenesis inhibition			
		Cm^r	Su^r	Sm^r	Sp^r	Km^r	Gm^r	Tm^r		Carrot disk	Sun-flower	Kalan-chöe	Tomato
pSa	–	+	+	+	+	+	+	+	100	+	+	+	+
pSa114	15.1–17.1	+	–	–	–	+	+	+	62	+	+	+	+
pSa134	14.4–17.4	+	–	–	–	+	+	+	46	+	+	+	+
pSa136	11.3–17.4	–	–	–	–	+	+	+	40	+	+	+	+
pSa200	9.5–12.7	–	+	+	+	+	+	+	115	+	+	+	+
pSa145	0–10.0	+	+	+	+	+	+	+	<2	–	–	–	–
pSa151	0–16.3	–	–	+	+	+	+	+	<2	–	–	–	–
pSa151-1	0–16.3 16.9–17.6	–	–	–	–	+	+	+	NT	NT	NT	NT	NT
pSa151-2	0–15.3, 17.9–18.5	–	–	+	+	–	–	–	NT	NT	NT	NT	NT
pSa201	0–12.7, 22.2–29.6	–	+	+	+	+	+	+	<2	–	–	–	–
pSa300	0–15.5, 24.6–29.6	–	–	+	+	+	+	+	<2	NT	NT	NT	NT
	Region cloned												
pBR322	–	–	–	–	–	–	–	–	NT	NT	NT	NT	NT
pBE1	17.1–19.2	–	–	–	–	+	+	+	NT	NT	NT	NT	NT
pHH1	11.3–14.4	+	–	–	–	–	–	–	NT	NT	NT	NT	NT
pBH5	14.4–19.2	–	+	+	+	+	+	+	NT	NT	NT	NT	NT
pBH6	16.9–19.2	–	–	+	+	+	+	+	NT	NT	NT	NT	NT

[a] Numbers refer to the linear map of pSa shown in Fig. 2

[b] *Abbreviations:* Cm, chloramphenicol; Su, sulfonamide; Sm, streptomycin; Sp, spectinomycin; Km, kanamycin; Gm, gentamycin; Tm, tobramycin; NT = not tested

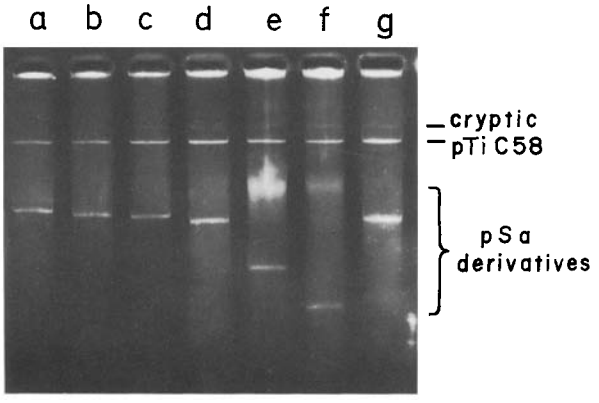

Fig. 5. Analysis of plasmids in *A. tumefaciens* transconjugants. pSa and six deletion derivatives were transferred to NT1 Rif^r Ery^r(pTiC58 Tra^c) as described in Materials and Methods. Plasmid DNA from each transconjugant was isolated and analyzed by gel electrophoresis. Lane a) pSa; lane b) pSa114; lane c) pSa134; lane d) pSa136; lane e) pSa145; lane f) pSa151; lane g) pSa200

ized with respect to their antibiotic resistance phenotype, efficiency of conjugation, and ability to inhibit oncogenesis in *A. tumefaciens*. Antibiotic resistance phenotype and efficiency of transfer were determined as described in Materials and Methods and the results are summarized in Table 1. Inhibition of oncogenesis was examined after conjugal transfer of the pSa derivatives to the oncogenic strain *A. tumefaciens* NT1 *Rif*^{r} *Ery*^{r} (pTiC58 Tra^c). After purification of the transconjugants, cultures were examined for the presence of plasmids. As can be seen in Fig. 5, each transconjugant contained the large cryptic plasmid and pTiC58 plasmid of *A. tumefaciens* in addition to a pSa derivative. These strains were inoculated on carrot discs, and on sunflower, *Kalanchoe* and tomato plants. Tumor formation was assayed as described in Materials and Methods. These results are also summarized in Table 1.

Discussion

By combining the information in Fig. 2 showing the location of the deletions in pSa with the information in Table 1 itemizing phenotypic changes associated with these deletions, it was possible to identify genetic functions on the restriction map of pSa (Fig. 6). For example, deletion of the region from 17.9 to 18.5 kbp on the pSa map (pSa151-2) inactivated Km^r, Tm^r, and Gm^r. In addition, the recombinant pBR322 plasmids containing this segment of DNA (pBE1, pBH5, and pBH6) conferred resistance to all three antibiotics. Finally, insertion of DNA fragments at the *Kpn*I site present at 17.8 kbp inactivated resistance to all three antibiotics (data not shown). These results indicate that Km^R, Tm^R, and Gm^R are encoded in this region and suggest that resistance may be determined by the same enzyme.

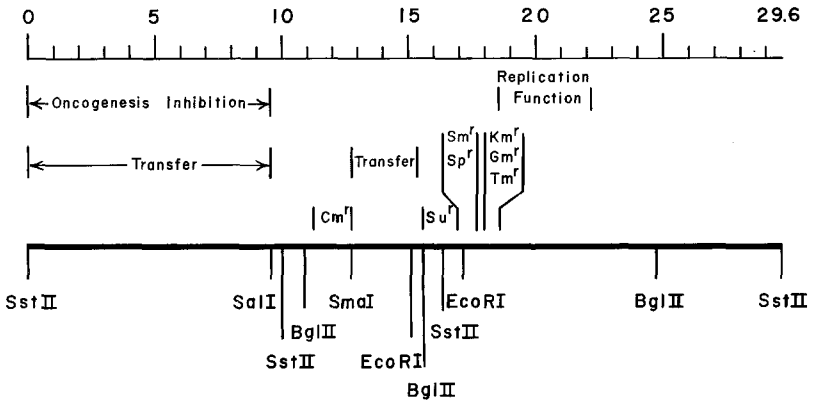

Fig. 6. Genetic map of pSa. The known genetic functions of pSa have been indicated on the restriction map of the plasmid. Four antibiotic resistance functions have been identified (Cm^r, Su^r, Sm-Sp^r, and Km-Gm-Tm^r), as have two regions involved in plasmid transfer. One of the transfer regions is also involved in the inhibition of oncogenesis. The maximum region necessary for efficient replication has been designated "Replication Function"

Several aminoglycoside-inactivating enzymes with similar broad specificity have been previously identified, including kanamycin acetyltransferase and gentamycin acetyltransferase (Benveniste and Davies 1971; Brzezinska et al. 1972). The plasmids pSa200 and pHH1 together define the minimum area involved in Cm^r, located at 11.3 to 12.7 kbp. This resistance in other R plasmids has previously been characterized as a type II chloramphenicol acetyltransferase activity (Gaffney and Foster 1978). As identifed by the derivatives pSa114, pSa134, pSa136, pSa151, pSa300, and pHB5, the minimum region required for Su^r has been determined to be 15.5 to 16.9 kbp. Although the nature of the Su^r encoded by pSa has not been determined, previous observations with Su^r mechanisms suggest alterations in membrane permeability as the most likely basis for plasmid encoded Su^r mechanisms (Watanabe 1963). The location of Sm^r and Sp^r has been identified from the plasmids pSa114, pSa151, pSa151-1 and pBE1. Resistance to both antibiotics is encoded from 16.9 to 17.6 kbp. Our observations indicate that insertion of DNA fragments in the *Eco*RI site at 17.1 kbp will inactivate both Sm^r and Sp^r without affecting Km^r (data not shown), suggesting that resistance to both Sm and Sp may be determined by the same mechanism. This has been previously observed with plasmid-mediated resistance to Sm and Sp, and the enzyme responsible for inactivating both antibiotics in other R plasmids has been characterized as streptomycin-spectinomycin adenylytransferase (Benveniste et al. 1970; Smith et al. 1970).

The deletion derivatives of pSa shown in Fig. 2 collectively encompass all but 4 kbp of the plasmid, suggesting that any pSa encoded information required for replication of the plasmid is contained within the region from 18.5 to 22.2 kbp. On the genetic map shown in Fig. 6, this area has been designated "Replication Function" and represents the maximum amount of pSa DNA necessary for efficient replication of the plasmid in both *E. coli* and *A. tumefaciens*. Subsequent results have identified within this 4 kbp region a 1.6 kbp fragment capable of autonomous replication in *E. coli* and a 0.47 kbp fragment of DNA containing the origin of replication (T. Close and R. Tait, unpublished observations).

Two regions of pSa have been identified that appear to be involved in the conjugal transfer of the plasmid. Deletions located in the region from 12.7 to 15.3 kbp reduced the efficiency of plasmid transfer by 40 to 60%. On the other hand, deletion of the region from 0 to 9.5 kbp reduced the efficiency of plasmid transfer to less than 2% of the frequency of wild-type pSa transfer. The importance of the 0 to 9.5 kbp region for conjugation is supported by studies of the plasmid pSa-1. This plasmid, which was constructed by insertion of the transposable element Tn1 into pSa, is defective in conjugal transfer (Hedges and Jacob 1974) and does not synthesize W pili (Bradley and Cohen 1976). Heteroduplex studies of pSa and pSa-1 (Gorai et al. 1979) and restriction endonuclease mapping of pSa-1 (data not shown) indicate the point of insertion of Tn1 as 0.5 kbp on the linear map of pSa.

The deletion in pSa200 shows that the two transfer regions at 0 to 9.5 kbp and 12.7 to 15.3 kbp are separated by 3.2 kbp of DNA that is definitely not required for efficient conjugation, but rather encodes Cm^r (Fig. 2, Table 1). We have observed that unselected cultures containing pSa will give rise to spontaneous Cm^s pSa derivatives. Previous results suggested that these derivatives were the result of spontaneous deletion events (Farrand et al. 1981). We examined three such derivatives, and all contained deletions in the region 10.5 to 14.5 kbp (data not shown), the same region removed in pSa200. Attempts to detect transposition of the Cm^r function of pSa were fruitless, suggesting that pSa does not contain a functional Cm^r transposable element. It should be noted that the Cm^R transposable element Tn9 gives rise to Cm^S deletion derivatives at a frequency of 10^{-4} to 10^{-3} (Federoff 1978, 1979). Likewise, the Cm^R determinant in pSa gives rise to Cm^S deletion derivatives at similar frequencies. Perhaps a Tn9-like element might be functioning in pSa.

Derivatives of pSa no longer capable of inhibiting crown gall oncogenesis all have deletions of the region 0 to 9.5 kbp, suggesting that information encoded within this region is involved in the inhibition phenomenon. It is possible that plasmid transfer and the suppression of crown gall oncogenesis are related. For example, the presence of the R factor may modify surface structures involved in the binding of the bacterium to the plant cell (Whatley et al. 1976; Mathysee et al. 1978), or may inhibit transfer of Ti plasmid DNA to the plant cell. Due to the lack of restriction endonuclease cleavage sites in the 0 to 9.5 kbp region of the plasmid, more precise resolution of the oncogenic suppressor region was not possible. We attempted to mutagenize this region by the insertion of Tn3, but of 11 independent Tn3 derivatives of pSa, all occurred within 0.05 kbp of the site of insertion of Tn1 in pSa-1 (data not shown). Apparently there is a high preference insertional site for Tn3 in pSa. We are currently attempting to use transposition of Tn10 and in vitro insertional inactivation (Heffron et al. 1978) to more precisely map the transfer and oncogenesis

inhibition functions and determine the relationship, if any, between the two phenotypes.

It has been suggested that pSa may constitute an ideal vector for cloning in oncogenic strains of *A. tumefaciens* (DeWilde et al. 1978). Discovery of the oncogenesis inhibition phenotype associated with pSa (Loper and Kado 1979; Farrand et al. 1981) made obvious an aspect of pSa that precluded its use as a vector. However, the demonstration that tumor inhibition phenotype can be separated from the replication and antibiotic resistance functions of pSa suggests that certain deletion derivatives of the plasmid may be useful in cloning and analysis of Ti plasmid DNA in *A. tumefaciens*. For example, plasmid pSa151 will replicate in *E. coli* and *A. tumefaciens*, can be used in either transformation or conjugation systems, can be selected on the basis of resistance to Sm, Sp, Km, Gm, or Tm, and possesses unique *Sst*II, *Eco*RI, *Bam*HI, *Pvu*II, and *Bgl*II sites for insertion of DNA.

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