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Relatedness of Strains of the B6 Group of Agrobacterium Tumefaciens Showing Altered Host Specificity

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RELATEDNESS OF STRAINS OF THE B6 GROUP OF *AGROBACTERIUM TUMEFACIENS* SHOWING ALTERED HOST SPECIFICITY

by

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VITA

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°C	degrees Centigrade
cfu	colony forming units
cpm	counts per minute
cRNA	complementary RNA
dal	dalton
DNA	deoxyribonucleic acid
g	gram
h	hour
i.e.	example
l	liter
M	molar
Mdal	megadalton
mg	milligram
min	minute
ml	milliliter
nm	nanometer
PB	phosphate buffer
pfu	plaque forming units
psi	pounds per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TCA	trichloroacetic acid
Ti-	tumor-inducing
TIP	tumor inducing principle
v/v	volume to volume
x g	times gravity

INTRODUCTION

When a plant wound site is infected with the gram negative soil bacterium Agrobacterium tumefaciens, contiguous plant cells are transformed into tumor cells exhibiting nonself-limiting, apolar cell division. The tumors, called crown gall, represent a disease occurring in 124 genera of dicotyledonous plants, including 16 genera of gymnosperms, and 2 genera of monocots (Lippincott and Lippincott, 1975).

Crown gall tumor tissue can be excised from an infected plant and grown on artificial medium free from viable bacterial cells (Braun, 1943). The tissue maintains its characteristic uncontrolled, disorganized growth pattern in the absence of auxin and cytokinin, two plant growth hormones required by normal plant tissue in culture.

Cultured normal tissue has not been successfully grafted onto a host plant. Cultured tumor tissue, however, when grafted back onto the original host plant or onto closely related plant species gives rise to a characteristic crown gall tumor. The grafted tumor tissue probably does not transform stock tissue, but merely proliferates. Crown gall tissue continues to demonstrate growth hormone independence and graftability after years of passage in cell culture. Apparently the inciting bacteria have induced some stable change in certain plant cells which results in their hormone-independent tumor development (Braun, 1943).

A Tumor-Inducing Principle

It is not understood how A. tumefaciens transforms normal tissue to crown gall tissue. Bacterial cells are not found within whole trans-

formed plant cells (Tourneur and Morel, 1970). Agrobacterium can occasionally be found inside terminally wounded cells at the inoculation site (Lippincott and Lippincott, 1976), but mainly exists intercellularly in plant tissue (Stonier, 1956). It has been hypothesized that the bacteria secrete some tumor inducing substance which causes a stable change to take place in the growth and organizational capacity of the susceptible plant. Much research has been directed toward identifying this "tumor inducing principle" (TIP, Braun, 1947).

Secondary tumors can arise on a host plant at distant points from tumors formed at the inoculated wound site. Agrobacterium has been known to travel internally in many plants, mainly via tracheids, for long distances (Lehoczky, 1968). This movement was concluded to account for secondary tumor formation in some plant hosts (Lippincott and Lippincott, 1976), however, secondary tumors are often free of detectable bacteria (White and Braun, 1941, 1942; Braun and Stonier, 1958). It is not clear in these cases that the TIP responsible for the secondary tumors came from the bacteria; it may have been elaborated by transformed plant cells at the primary site. In either case the appearance of a tumor at a point distant from the inoculation site suggests that the TIP is a transmissible substance (White and Braun, 1942; Lippincott and Lippincott, 1975). Because metastasis is not seen in plants it is unlikely that such secondary tumors arise from movement of cells forming the primary tumor.

Host Cell Transformation: A Two Step Process

An in vivo study of the factors involved in tumor formation was

undertaken by Braun and Laskaris (1942) using an attenuated strain of A. tumefaciens. This strain was normally able to incite only a slight proliferation of cells at a tomato plant wound site. Host cells were altered by the bacterial strain, but the cell growth rate did not become uncontrolled as is characteristic of crown gall tumor cells (Braun and Laskaris, 1942). Only after auxin was supplied to stimulate the transformed plant cells, did growth rates increase and large transplantable tumors result. This work indicated a need to distinguish between factors that make cells neoplastic and factors that affect their subsequent development (Braun, 1943).

Growth of normal tobacco pith parenchymal cells in vivo and in vitro proceeds in a two step fashion. When treated in vitro with a synthetic auxin, such as naphthalene acetic acid, freshly isolated pith cells increase rapidly in size but do not divide. Only when another growth substance, such as 6-furfuryl-amino purine (a cytokinin), is supplied with the auxin is growth followed by cell division. When healthy pith cells are converted to crown gall tumor cells both growth substances are synthesized by the plant cells in greater than regulatory amounts (Braun, 1958). The basic difference between a normal cell and a tumor cell appears to be at the physiological level. Activation of growth factor synthetic systems, whose products are concerned with growth and cell division, takes place (Braun, 1958). How the TIP associated with this disease accomplishes the alteration of host metabolic systems remains unknown.

Candidates for TIP

Nearly every major cellular component of A. tumefaciens has been implicated or suspected as TIP and examined for tumor-inducing ability. These components include: 1. products of bacterial metabolism, (Kado, 1976); 2. a chemical fraction of the bacterial cell such as DNA, (Kovoor, 1967), RNA (Beljanski et al., 1974; Swain et al., 1972) or protein, (Kado, 1976); 3. bacteriophages associated with A. tumefaciens (Leff and Beardsley, 1970) and 4. cell wall material (i.e. endotoxin, Savulesco et al., 1971). The whole bacterial cell itself has been suspected of causing crown gall tumors. Since bacterial cells are never found within plant cells it was thought that the A. tumefaciens cell could have become drastically altered morphologically and physiologically so that it was no longer demonstrable by current isolation techniques (D'Herelle and Peyre, 1927). Support for the altered bacterial cell hypothesis comes from the known examples of Rhizobium species which form pleomorphic bodies in cells of root nodules of legumes (Kado, 1976). Beardsley's (1972) attempts to demonstrate the presence of an L-form in cultures of S8 sunflower tumor tissue were repeatedly unsuccessful. Rubio-Huertos and Beltra (1962) have reported stable L-forms that were tumorigenic but these results have been disputed (Cabezas de Herrera and Rubio-Huertos, 1967; Beardsley et al., 1966). It was also considered possible that the bacteria converted a normal host cell constituent into the TIP (Kado, 1976).

Kado (1976) has suggested that the TIP should be an "autoreplicating epigenetic factor that conferred abnormal regulatory growth characteristics on a normal cell." That is, the pattern of gene expres-

sion had changed through epigenetic mechanisms. Genetic mechanisms in which a gain, loss or rearrangement of cellular genetic information takes place could also explain the tumor cell characteristics. The major candidate for TIP has been the nucleic acids because of their self-replicating potential and their ability to induce a stable change in cell phenotype. Transfer and integration of genetic material would explain how sterile cultured tumor tissue maintains its uncontrolled, disoriented growth pattern in vitro and when it is grafted back to a susceptible host. Attempts to justify this choice for TIP have yielded conflicting reports. Bacterial DNA preparations from A. tumefaciens are reportedly capable of inducing tumors (Kovoor, 1967), but this report has not been confirmed (Phillips and Butcher, 1975). Plant tissue transformation by RNA from A. tumefaciens (Swain, 1972) and isolation of tumor-inducing RNA from A. tumefaciens (Beljanski, 1974) have also been reported.

In 1955, Beardsley discovered the phenomenon of lysogeny in A. tumefaciens. Bacteriophage PS8 (an omega type phage, DeLey et al., 1972; Schell, 1975) has been identified as a standard phage of the B6 group. Many studies have been conducted in an effort to firmly link lysogeny to tumorigenicity. Several laboratories have demonstrated that phage activity was present in homogenates of sterile crown gall tissue (Klein and Beardsley, 1957; Parsons and Beardsley, 1968; Tourneur and Morel, 1970). Following these reports Leff and Beardsley (1970) reported that PS8 DNA preparations were able to induce tumor formation on healthy plant tissue. In 1973, Schilperoort et al. reported the presence of phage PS8 DNA base sequences in sterile crown gall

tissue by DNA:DNA hybridization experiments. These workers were, however, unable to detect free viable phage particles in crown gall tissue homogenates. In addition, the presence of phage particles in homogenates of crown gall tissue has not been verified by electron microscopic observation (Schilperoort et al., 1973). The oncogenic activity of the phage has been disputed (Biederbeck et al., 1973) since the same phage have been observed in both virulent and avirulent Agrobacterium strains (Kado, 1976). Many tumorigenic strains harbor no omega phage (Schell, 1975) and strains cured of endogenous phage are as pathogenic as the parent strain (De Ley et al., 1972).

The demonstration of bacterial nucleic acids in tumor tissue would link those nucleic acids to TIP. Partial homology has been reported between A. tumefaciens bacterial DNA and tumor cell DNA (Quetier et al., 1969) and between complementary RNA transcribed from A. tumefaciens DNA and crown gall tumor DNA (Schilperoort et al., 1967). Other investigators, however, have been unable to detect crown gall tumor DNA sequences homologous to A. tumefaciens DNA or bacteriophage PS8 DNA (Chilton et al., 1974a; Drlica and Kado, 1974; Eden et al., 1974; Farrand et al., 1975). The experiments by Chilton et al. (1974a) would have detected one genome copy per diploid tumor cell. However, the presence of a unique nucleotide segment smaller than about 5% of the bacterial or bacteriophage genome would not have been detected, even if present in multiple copies per tumor cell. It is possible that a few specific virulence genes could have been transferred from the bacteria to plant cells and remained undetected by these investigations.

The Role of Plasmid DNA in Tumor Induction

When the highly virulent strain of A. tumefaciens strain C58 is cultured at 36°C, the ability to induce crown gall tumors is lost (Hamilton and Fall, 1971). The conversion of strain C58 to the avirulent state occurs at too high a frequency to be explained by heat induced mutation. The event is better explained by loss of an autonomous virulence factor or plasmid. This hypothesis was tested by Van Larebeke in 1974. A broth culture of strain C58 was incubated at 37°C. At timed intervals a sample was withdrawn, plated at 28°C, and random colonies from each sample were tested for tumorigenicity. Both tumorigenic and nontumorigenic colonies were identified and screened for the presence of a plasmid. It was found in all cases that tumorigenic colonies harbored a plasmid while non-tumorigenic colonies had no plasmid. The stable conversion to nonpathogenicity was apparently accompanied by loss of a plasmid.

Zaenen et al. (1974) systematically screened a number of virulent and avirulent Agrobacterium strains for the presence of plasmid DNA. These workers found that a large plasmid was present in the eleven pathogenic strains analyzed. None of the eight avirulent strains examined contained such a plasmid.

Pathogenicity is a characteristic that can be gained by a bacterial strain. In 1969 Kerr reported the apparent transfer of tumorigenicity between strains of Agrobacterium in plants. Tumors were induced on a tomato plant by a virulent Agrobacterium (sp.) donor. The resulting crown galls were inoculated with an avirulent chloramphenicol resistant Agrobacterium recipient. After several weeks incubation the

recipient species was isolated from the tumor tissue and shown to be pathogenic. This was the first unequivocal evidence for transfer of virulence between isolates of Agrobacterium although there was no indication of the mechanism of transfer.

Hamilton and Chopan (1975) performed a similar experiment. They induced tumors on the stems of tomato plants and then inoculated the galls with a polymyxin resistant, cured derivative of C58. After two weeks the tumors were excised, ground in a mortar and dilutions of the tumor homogenate plated on selective media. Five out of fourteen tumors were found to contain polymyxin resistant virulent bacteria. This showed that the tumor-inducing substance could be removed from and reintroduced into a strain of A. tumefaciens by a process that could occur in nature.

It was considered possible that this phenomenon might be explained by plasmid transfer and subsequent work proved this to be true. Tumor-inducing ability is acquired by nontumorigenic agrobacteria as a result of the acquisition of plasmid DNA from tumorigenic donor strains (Van Larebeke et al., 1975; Watson et al., 1975; Chilton et al., 1976). Similar results have been obtained with several different A. tumefaciens donor strain including C58, K27, 223, 0362 and EU6. There is evidence that the Ti-plasmid is a conjugative plasmid (Genetello et al., 1977) and that transfer of virulence occurs in vivo and in vitro in Agrobacterium (Kerr et al., 1977).

Additional Characteristics of the Virulence Plasmid

All tumorigenic strains of A. tumefaciens examined harbor at least

one covalently closed circular DNA plasmid (Currier and Nester, 1976a; Van Montagu and Schell, 1975; Zaenen et al., 1974). This element called the Ti-plasmid, appears to play a key role in determining virulence (Van Larebeke et al., 1974, 1975; Watson et al., 1975; Zaenen et al., 1974). Engler et al. (1975) noted that it is essential to have known genetic markers on the plasmid if one is to study its role in the conversion of normal plant cells to crown gall tumor cells. Subsequently additional characteristics of the virulence plasmid were identified.

The sensitivity some oncogenic A. tumefaciens strains exhibit to a bacteriocin elaborated by A. radiobacter strain 84 (Kerr and Htay, 1974) is determined by the Ti-plasmid (Kerr and Roberts, 1976). Exposure of sensitive strains to agrocin 84 selects resistant colonies and these resistant colonies are found to no longer be pathogenic (Kerr and Htay, 1974). Engler et al. (1975) confirmed these observations and further showed that the virulent agrocin 84 sensitive strain C58 becomes resistant to the action of the agrocin when cured of the Ti-plasmid by cultivation at 37°C and that agrocin 84 resistant colonies have simultaneously lost tumor inducing ability and the Ti-plasmid. This work indicated that both tumor inducing ability and sensitivity to agrocin 84 were controlled by genes carried by the Ti-plasmid (Engler et al., 1975). The genes determining agrocin 84 sensitivity are probably not necessary for oncogenicity, however, since not all virulent A. tumefaciens strains are sensitive to this bacteriocin (Engler et al., 1975).

Crown gall disease has been controlled under greenhouse conditions by inoculation of susceptible hosts with the nonpathogenic strain 84 (Kerr, 1972; New and Kerr, 1972). One experiment, in which a 1:1 mixture

of strain 84 and a pathogen was applied to a plant, resulted in nearly complete suppression of gall formation by 6 out of 8 pathogens (New and Kerr, 1972). In the other 2 cases tumors formed were significantly reduced in size. The method by which agrocin 84 prevents tumor formation has been studied by Smith and Hindley (1978) who suggest that the bacteriocin acts by interrupting cell wall synthesis leading to fragmentation or loss of cell envelope. This action impairs the attachment of the bacterium to the host cell and the subsequent transfer of DNA to the host. These workers further suggest that agrocin sensitivity is based on bacterial nutrition and report that media supporting capsular lipopolysaccharide synthesis also inhibits agrocin 84 sensitivity completely. It is believed that the surface components which are involved in bacterial attachment to host are capsular lipopolysaccharides (Smith and Hindley, 1978).

The agrobacteriophage AP1 has a broad host range (Hernalsteens et al., 1978). It has been reported that the Agrobacterium Ti-plasmid from strain B6S3 enables its host bacteria to exclude this virulent phage (Van Larebeke et al., 1975, 1977; Schell, 1975). Surveys indicated that phage AP1 is able to adsorb to most strains of Agrobacterium, but only plaques on strains lacking the Ti-plasmid (Schell, 1975). That the phage forms plaques on agrocin 84 resistant mutants, but not on agrocin 84 sensitive strains, is strong evidence that AP1 exclusion is a Ti-plasmid coded function (Schell, 1975).

Most pathogenic strains of Agrobacterium are able to utilize either octopine or nopaline, unusual non-proteinaceous guanido-amino acids (Lippincott et al., 1973; Petit et al., 1970; Bomhoff et al.,

1976; Hooykaas et al., 1977) while most, but not all, avirulent strains fail to degrade either of these compounds (Lippincott et al., 1973; Merlo and Nester, 1977). Petit and Tourneur (1972) reported that loss of tumorigenicity in strain B6 was accompanied by loss of the ability to degrade octopine. This indicated that the ability to utilize these amino acids is a plasmid borne trait. Later studies by Bomhoff et al. (1976) clearly demonstrated that the specificity and activity of the enzyme-systems for octopine or nopaline utilization in A. tumefaciens strains were coded for by the Ti-plasmid. This conclusion was based on two observations. First, when a nopaline or octopine degrading strain was cured of its Ti-plasmid, the strain was unable to utilize nopaline or octopine as sole nitrogen source. Second, when the Ti-plasmid of a nopaline degrading strain was transferred into a plasmid-free avirulent recipient, the virulent transconjugant was found to have gained the ability to utilize nopaline. It has been proposed that octopine or nopaline utilization could be used instead of virulence testing to screen cultures for cells which harbor the Ti-plasmid (Kerr, 1975).

The unusual amino acid derivatives octopine and nopaline are detectable in crown gall tissue (Menage and Morel, 1964, 1965), but not in normal plant tissue (Menage and Morel, 1964; Montoya et al., 1977; Kemp, 1976). It has been demonstrated that nopaline utilizing strains induce nopaline producing crown gall tissue and likewise octopine utilizing strains induce octopine utilizing crown galls (Bomhoff et al., 1976; Goldmann et al., 1969; Petit et al., 1970). Strains that do not fit this description include the nopaline utilizers that induce tumors that synthesize neither nopaline nor octopine and strains able to uti-

lize octopine and nopaline, but which induce tumors producing only nopaline (Montoya et al., 1977).

The strains that utilize both octopine and nopaline were examined more closely. When plasmid DNA isolated from these strains was used to transform plasmidless recipients, the transformants isolated were virulent and able to utilize nopaline. The octopine utilization trait was not transferred. The same observation was made when the plasmid was transferred by in planta conjugation. In these strains it appears that the nopaline utilization trait is plasmid-borne while the octopine utilization trait is not coded for by the plasmid (Montoya et al., 1977). Further experimentation has led these workers to tentatively conclude that in A. tumefaciens strains which utilize both octopine and nopaline the utilization of octopine is coded by chromosomal genes (Montoya et al., 1978).

The synthesis of either octopine or nopaline in crown gall cells also appears determined by genes located on the Ti-plasmid. Mutants have been isolated from nopaline utilizing and octopine utilizing strains which no longer degrade nopaline or octopine. These mutants induced tumors which produced the same relative amount of nopaline or octopine as tumors induced by the parental strains (Montoya et al., 1977). This suggests that two distinct plasmid borne gene sets exist, one determining synthesis of octopine or nopaline by the tumor and one determining utilization of the amino acid by the bacteria.

Two additional observations have been made regarding the guanidoamino acids. Lippincott and Lippincott (1970) showed that small amounts of octopine promoted growth of crown gall tumors. They observed a

linear relationship between tumor diameter and the logarithm of the concentration of octopine applied to an infected leaf. Octopine was not active in the tumor initiation process (Lippincott and Lippincott, 1969); that is the amino acid had no effect on the number of tumors induced, only on the rate at which they grew. It also appeared that the opines promoted Ti-plasmid conjugation (Genetello et al., 1977; Petit et al., 1978; Kerr et al., 1977). Both catabolic activity and plasmid transfer activity were induced by the presence of octopine, octopinic acid or lysopine. These compounds were substrates of enzymes coded for by the Ti-plasmid. These results support the hypothesis that a common regulatory gene controls the two distinct operons for catabolic activity and plasmid transfer (Petit et al., 1978).

Detection of Plasmid DNA in Crown Gall Tumor Tissue

While virulent strains of A. tumefaciens often harbor more than one plasmid, not all large plasmids determine virulence. Only one type, the Ti-plasmid, is necessary for tumor induction. Evidence has been discussed supporting the essential role of the Ti-plasmid in tumorigenicity (Van Larebeke et al., 1974, 1975; Watson et al., 1975; Zaenen et al., 1974). None of this evidence explains how the plasmid effects the transformation of a normal host cell to a tumor cell. The following observations suggest that the plasmid or a portion of it may be transferred to the host cells. First, crown gall cells maintain their tumor characteristics for years in tissue culture (Braun, 1958; Lippincott and Lippincott, 1976). Second, tumor tissue produces octopine or nopaline in tissue culture (Montoya et al., 1977). Third, the ability to

synthesize octopine or nopaline is a plasmid borne trait (Petit and Tourneur, 1972; Bomhoff et al., 1976).

Initial attempts to detect Ti-plasmid DNA sequences in tumor tissue were unsuccessful or the successful results were not reproducible. Using radiolabeled plasmid DNA in solution renaturation experiments, workers were unable to detect homologous sequences in crown gall DNA (Chilton et al., 1974b). It was concluded that sequences homologous to the entire Ti-plasmid were not present in the tumor (Chilton et al., 1974b; Dons, 1975; Gordon et al., 1976). These experiments were performed under conditions which would have detected one plasmid copy per diploid tumor cell genome. However, a specific set of sequences representing less than 5% of the plasmid would not have been detected.

In 1976 DNA hybridization studies were conducted by Matthysse and Stump to examine the possible role of plasmid DNA in tumor induction. This work detected DNA complementary to A. tumefaciens plasmid in bacterium-free Vinca rosea tumor tissue induced by strain A6. Plasmid DNA composed roughly 0.1% of the tumor cell DNA. These results, however, have not been successfully reproduced (Farrand, personal communication; Gordon et al., 1976; Sciaky, 1977).

Chilton et al. (1977a) repeated the renaturation kinetic analyses with several refinements designed to increase the sensitivity of the tests. Since the 1974 study, which used strain B6-806 radiolabeled plasmid as the probe, strain B6-806 was shown to harbor two plasmids. A strain, A277, was constructed which contained only the B6-806 Ti-plasmid. The Ti-plasmid from this strain was used as radiolabeled

probe. Second, unlabeled DNA from single-cell-cloned crown gall tumor tissue was used in renaturation kinetic study. When Chilton et al. (1977) allowed radiolabeled pTi-A277 probe to reanneal in the presence of excess unlabeled cloned tobacco tumor DNA, a slight acceleration of probe reassociation was observed. These results were reproducible and suggested that Ti-plasmid sequences might be present in tobacco tumor DNA. Control experiments indicated that less than one copy of the entire plasmid was present per diploid tumor cell so either plasmid DNA was present at very low levels or only part of the plasmid was present in the tumor DNA (Chilton et al., 1977).

Radiolabeled plasmid DNA was digested with restriction endonuclease SmaI and the DNA fragments were separated according to molecular weight by agarose gel electrophoresis. Fragment bands were extracted from the gel and each allowed to reassociate in the presence of cloned tumor DNA. If the whole plasmid was present, reassociation of all bands should be accelerated by the presence of tumor DNA. However, only the bands 3b and 10 showed a change in reassociation kinetics in the presence of tumor DNA. This change was not observed when the bands were allowed to reassociate with normal tobacco DNA. The data obtained indicated that about 20 copies of band 3b, from the SmaI digested Ti-plasmid DNA, were present per tumor cell (Chilton et al., 1977).

Plasmid Bands 3b and 10c Detected in Crown Gall Tumors are Contiguous

Renaturation of the two plasmid bands 3b and 10c, described above, was accelerated in the presence of tumor DNA. It was possible that

these two fragments were contiguous on the Ti-plasmid geonome and a restriction endonuclease site located within this region led to their separation. A second possibility was that each band was located in a distinct region of the plasmid genome and each was transferred separately into the host plant cell. Chilton et al. (1978a) constructed a physical map of pTi-B6-806 from restriction endonuclease SmaI and HpaII digests. The two SmaI restriction fragments contained in bands 3b and 10c were contiguous on the pTi-B6-806 map.

Plasmid DNA is Transcribed in Crown Gall Tumors

While it is clear that plasmid DNA sequences are present in tumor cells, it is unknown how this information effects the transformation of a normal cell to a tumor cell. In a step to clarify the mode of transformation, radiolabeled RNA from a cloned tobacco tumor line was isolated and hybridized with banded fragments of restriction endonuclease SmaI digested pTi-B6-806. Radiolabeled tumor cell RNA hybridized to only band 3b of the SmaI digest, (Drummond et al., 1977). The data confirmed the presence of specific fragments of the Ti-plasmid in tumor cells and demonstrated that foreign DNA of bacterial origin could be transcribed in plant tissue. Not all of the foreign DNA was transcribed at detectable levels, but at least some transcription was demonstrated by this technique (Drummond et al., 1977).

Detection of plasmid DNA sequences in plant tumors implicated the Ti-plasmid as the TIP. It is tempting to propose that these incorporated segments of DNA code for functions involved in tumorigenicity. This work was significant for several reasons (Chilton et al., 1977;

Sciaky, 1977), first, it is the only case of stable incorporation of bacterial genes in any eukaryotic cell and second, it provides a model system for genetic engineering in plants. Crown gall disease may be established as a model system for understanding the molecular basis of viral induced animal cancer.

The Ti-plasmids: Extent of Homology

If crown gall is a unified disease with a single common cause, i.e. the Ti-plasmid, then the Ti-plasmids from all A. tumefaciens strains might be expected to show high sequence homology. Currier and Nester (1976a) determined the base sequence homology of plasmids isolated from various strains of A. tumefaciens. They identified two genetically distinct classes of plasmids. One class was closely related to the plasmid of strain A6, an octopine utilizer. The other class was related to the plasmid of strain C58, a nopaline utilizer. There were also several strains that did not belong to either of the two groups. Plasmid homology within the group of octopine-utilizers was high (74-100%) while that within the group of nopaline utilizers was divergent (28-97%). When plasmids of the different groups were compared, less than 30% homology, at best, was found.

Sciaky et al. (1978) surveyed Ti-plasmids of Agrobacterium to confirm and extend Currier and Nester's findings (1976a). Purified Ti-plasmids were digested with restriction endonuclease SmaI and the fragments were separated by slab gel electrophoresis. The number of bands distinguished in each preparation and the relative molecular weight of each band was compared. Based on this information and the

genetic characteristics of the Ti-plasmids Sciaky et al. (1978) divided the plasmids studied into three classes. One class, the octopine type Ti-plasmids, conferred pathogenicity and the ability to utilize octopine. These plasmids were found to be nearly identical by restriction endonuclease analysis. The second plasmid class, the nopaline type Ti-plasmids, determined pathogenicity and the ability to utilize nopaline. These Ti-plasmids were found to show a wide range of relatedness. The third class of plasmids, null type, conferred tumorigenicity, but did not enable the bacterium to utilize octopine or nopaline. At this time the third group contains only one member. The plasmid restriction endonuclease fragment pattern is complex due to the presence of multiple plasmids. When the octopine type and nopaline type plasmids were compared they were found to differ greatly from one another. Only one fragment band appeared to be common to octopine and nopaline type plasmids. These findings parallel those of Currier and Nester (1976a).

The Ti-plasmids of Agrobacterium may be octopine type-, nopaline type-, or null type plasmids. It has been reported that the base sequence homology between these Ti-plasmid classes may be as little as 5% (Currier and Nester, 1976a; Sciaky et al., 1978) and that diverse plasmids show only one fragment band in common after digestion with SmaI (Sciaky et al., 1978). This evidence suggests that if virulence genes from various Ti-plasmids are closely related, they can compose only a small fraction of the total plasmid genome.

Since the identification of strain B6-806 Ti-plasmid (pTi-B6-806) sequences in crown gall cells, much work has concentrated on mapping

the plasmid location of the segment maintained in tumor cells (T-DNA, Chilton et al., 1978a) and searching for related sequences on the diverse types of Ti-plasmids. Data has been presented which show that a 5.5 Mdal segment of DNA is highly conserved among different Ti-plasmids and that this segment overlaps the T-DNA of pTi-B6-806 (De Picker et al., 1978; Chilton et al., 1978b). However, not all sequences of the pTi-B6-806 T-DNA are common to the various Ti-plasmids studied (Chilton et al., 1978b). It has been suggested that the non-conserved T-DNA encodes pTI-B6-806 conferred octopine production by tumor cells, a trait which is not common to nopaline type plasmids (Chilton et al., 1978b). Furthermore, the work of DePicker et al. (1978) gives evidence that the common region of the T-DNA is essential for pathogenicity.

Two laboratories have suggested that the octopine type and nopaline type plasmids may be incompatible (Montoya et al., 1978; Kerr and Roberts, 1976). Montoya et al. (1978) were unable to transform strains carrying octopine type plasmids with nopaline plasmid DNA and vice versa. Further, these workers found that although null type plasmid strains could be transformed with octopine type or nopaline type plasmids, the transformed strains would not stably maintain the foreign Ti-plasmid. The incompatibility of the Ti-plasmids is unexpected considering their low level of sequence homology (Montoya et al., 1978).

Following up on this work, Drummond and Chilton (1978) compared pTi-B6-806, which confers the ability to utilize octopine, to several heterologous nopaline type plasmids in order to determine if the small highly conserved segment of T-DNA was the only region common to all

Ti-plasmids. Hybridization studies revealed that the Agrobacterium Ti-plasmids share extensive regions of DNA homology, up to 56% of pTi-B6-806, although the common region is more rigidly conserved than these other regions. As the common regions are separated by areas of non-conserved DNA Drummond and Chilton (1978) suggest that selective pressure accounts for the maintenance of the regions of homology found on all Ti-plasmids despite the recombination, insertion and deletion events that must have taken place.

Other Agrobacterium Plasmids

Zaenen et al. (1974) surveyed strains of Agrobacterium and found large plasmids in eleven tumorigenic strains and no plasmids in eight nontumorigenic strains. Merlo and Nester (1977) surveyed naturally occurring nonpathogenic strains for the presence of plasmids and for biochemical properties known to be associated with plasmids in virulent strains. Surprisingly, all nontumorigenic strains examined were found to harbor one or more plasmids. Several of these strains exhibited significant (30-60%) base sequence homology with the Ti-plasmid from the virulent strain C58 (Merlo and Nester, 1977). In addition, five nontumorigenic strains were able to metabolize nopaline and one strain was able to utilize both octopine and nopaline. Agrocin 84 sensitivity and the presence of a large plasmid were not correlated in the nonpathogenic strains (Merlo and Nester, 1977).

Currier and Nester (1976) worked with strain 27, a virulent strain, known to contain 2 plasmids. A virulent daughter strain was isolated which contained only the larger of the two plasmids. Although

the second plasmid was not essential for pathogenicity, it may also determine virulence, since it was stably carried by the daughter strain.

Nontumorigenic strains of Agrobacterium have been shown to harbor large plasmids. Tumorigenic strains of Agrobacterium have been demonstrated to contain plasmids other than the Ti-plasmid. The role of these plasmids and their relationship to the Ti-plasmids is not well understood.

Two Plasmids are Present in the Strain B6-806

Separate lines of evidence have implicated the presence of two plasmids in the standard strain B6-806.

Renaturation kinetic analysis was used to compare the plasmid content of donor strain B6-806 to one of its virulent transconjugants. The Ti-plasmid was transferred in vitro from strain B6-806 to a plasmidless Agrobacterium recipient, strain NT1. When solution hybridization of plasmid DNA from the transconjugant was performed with excess strain B6-806 plasmid DNA, 100% sequence homology was observed. However, when strain B6-806 plasmid DNA was hybridized with excess transconjugant plasmid DNA only 60% hybridization was observed (Farrand, personal communication). All of the DNA sequences from the transconjugant plasmid preparation were present in plasmids from strain B6-806. The strain B6-806 plasmid preparation, however, contains sequences that were not present in the transconjugant plasmid. A possible explanation for the difference in sequence homology of the two preparations was that strain B6-806 contained two plasmids only one of them

being transferred to strain NT1.

Plasmid preparations from strain B6-806 and two of its derivatives were compared after restriction endonuclease SmaI digestion and fragment separation by slab gel electrophoresis. One of the derivatives, strain A277, contains the Ti-plasmid of strain B6-806. The other derivative strain A217, is NTG-induced avirulent mutant of strain B6-806. The pattern of plasmid fragments from strain B6-806 on an agarose gel after SmaI digestion was complex (Sciaky, 1977). That of the transconjugant strain A277 was simple by comparison and appeared to represent a subset of the fragments from strain B6-806 (Sciaky, 1977). While approximately half of the plasmid fragments of strain B6-806 were absent in strain A277, the molecular weight of plasmid DNA from strain B6-806 and strain A277 do not differ to this extent. The pattern of plasmid fragments from strain A217 also appeared simple when compared to that of strain B6-806. When fragment patterns from the three strains were compared, an interesting relationship was observed. If the plasmid cleavage pattern of strain A277 and strain A217 were combined, the pattern of the parent, strain B6-806, was obtained. It is most likely that strain B6-806 contains two plasmids and strain A277 and strain A217 each contain one of the parental plasmids.

Plasmid Coded Host Specificity Markers

Some data exist suggesting that host specificity markers of strains of the B6 group of Agrobacterium may be associated with their plasmids. Four B6 derivatives were collected: B6-806 (a standard lab strain, isolated as a phage-sensitive derivative of B6), A217 (an

avirulent mutant of strain B6 that contains a large plasmid), strain B6-Braun (a culture of B6 obtained from A. C. Braun) and strain TR1WS (a strain with B6-group characteristics although it was cultured from a slant of A. radiobacter TR1). Strain B6-806 is virulent on Kalanchoe, carrot and sunflower while strains B6-Braun and TR1WS are virulent on carrot and sunflower but not on Kalanchoe. Strain A217 as described above, was isolated as an avirulent mutant of strain B6-806 (Sciaky, 1977; Sciaky et al., 1978).

A difference in the plasmid content of these four strains has been demonstrated. One strain with a limited host range, strain TR1WS, was found to contain chromosomal and plasmid DNA sequences that were 100% homologous with the chromosome and plasmid DNA of strain B6-806 (Farrand, personal communication). However, conflicting results were obtained when the reverse hybridizations were performed. Plasmid from strain B6-806 showed only 75% homology with plasmid from strain TR1WS (Farrand, personal communication) indicating that the strain B6-806 plasmid preparations contained some sequences that were absent from strain TR1WS. Subsequent reports indicated that two plasmids exist in strain B6-806 (Sciaky, 1977; Sciaky et al., 1978). One of the plasmids is the Ti-plasmid, pTi-B6-806. The other plasmid is cryptic and carries no known genetic markers, pAt-B6-806 (Sciaky, 1977). It is believed that strain A217 harbors only the cryptic plasmid, having been cured of its Ti-plasmid by nitrosoguanidine treatment (Sciaky et al., 1978). Perhaps differences in tumorigenicity could be attributed to differences in plasmid content.

A second explanation for the difference in demonstrated host

range is that several strains have lost host range genes through deletion of plasmid DNA. The virulence genes and postulated host range genes may reside on the same plasmid and a portion of this plasmid may have been deleted in a particular strain. If true, then host range is a characteristic of tumor induction that can be attributed directly to the Ti-plasmid.

Electron Microscopy of the A. tumefaciens Plasmids

Zaenen et al. (1974) reported the presence of large plasmids in various virulent strains of A. tumefaciens. These plasmids reportedly ranged from 95.8 x 10^6 dal to 156 x 10^6 dal in molecular mass. A later survey conducted by Currier and Nester (1976a) agreed with these values and revealed that many strains of Agrobacterium contained more than one plasmid size class. The A. tumefaciens strain 27 was found to harbor a 127 x 10^6 dal and a 153 x 10^6 dal plasmid (Currier and Nester, 1976a), for example. The strain B6-806 plasmid measurement was found to be 125 x 10^6 dal (Currier and Nester, 1976a). In the case of strain B6-806 two plasmid size classes were not identified although evidence of two different plasmids has been described (Sciaky et al., 1978). The pTi-B6-806 isolated from strain A277, which contains only the Ti-plasmid, has been measured to be 120 x 10^6 dal (Chilton et al., 1977a). The size of other B6 strain plasmids, including the pAt-B6-806, have not been individually determined. An electron microscopy study of the contour lengths of the large plasmids of the B6 strains may reveal a significant difference in their size.

Characterization of the B6 Strains

Although pathogenic A. tumefaciens strains often harbor more than one plasmid (Currier and Nester, 1974a; Sciaky et al., 1978), all plasmids harbored by a strain are not necessarily Ti-plasmids (Currier and Nester, 1976a). Some Agrobacterium plasmids do not code for any known functions. The origin of these cryptic plasmids is unknown and little study has been devoted to determining the role these plasmids play in host strains.

As described above, certain differences have been found among various derivatives of strain B6. For example, strains B6-806 is tumorigenic on a wide variety of hosts including carrot, sunflower, tobacco and Kalanchoe. Strains TR1WS and B6-Braun, on the other hand, are nontumorigenic on Kalanchoe. Strain B6-806 harbors two large plasmids, pTi-B6-806 and pAt-B6-806. Strain TR1WS appears to harbor only a single plasmid. Such differences would suggest that some genetic divergence has occurred among the B6 strains since the original isolation of the original strain in 1934.

A survey of 19 A. tumefaciens B6 strains collected from various laboratories was conducted in order to assess the extent of this divergence. Such a study was considered important in that experiments utilizing these strains are performed in many different laboratories and findings are often compared. It is possible that characterization of the B6 strains and comparison of the plasmid content could be valuable in evaluating the role of the plasmids in pathogenicity.

MATERIALS AND METHODS

Bacterial strains. A list of the bacterial strains used appears in Table 1. Most strains were the gifts of other researchers as indicated. Other strains were constructed in this laboratory.

Media. All strains were routinely grown in L-broth (Levin et al., 1976) containing in grams per liter: tryptone (Difco), 10; yeast extract (Difco), 5; and sodium chloride, 5. The pH was adjusted to 7.2-7.4 before the medium was autoclaved. Chalk agar (Bernaerts and De Ley) contained in grams per liter: yeast extract, 10; glucose, 20; calcium carbonate, 20; and agar, 20. Lactose agar (Bernaerts and De Ley) contained in grams per liter: lactose, 10; yeast extract, 1; and agar, 20. Basal medium (Kerr et al., 1977) contained in grams per liter: potassium phosphate, dibasic, 10.5; potassium phosphate, monobasic, 4.5; magnesium sulfate, 0.2; ferrous sulfate, 0.005; and manganese chloride, 0.002. AB minimal medium (Chilton et al., 1974a) contained in grams per liter: potassium phosphate, dibasic, 3; sodium phosphate, monobasic, 1; ammonium chloride, 1; magnesium sulfate, 0.3; potassium chloride, 0.15; calcium chloride, 0.01; and ferrous sulfate, 0.0025. Minimal medium contained either 0.5% glucose or 500 µg/ml octopine as a carbon source. Nutrient agar (NA) contained in grams per liter: nutrient broth (Difco), 8, and agar, 15. Soft nutrient agar contained in grams per liter: nutrient broth (Difco) 8, and agar, 7.

TABLE 1. *Agrobacterium tumefaciens* and *Escherichia coli* Strains Studied.

Strain	Description	Source
B6-806	Bacteriophage PS8 sensitive derivative of B6 isolated after repeated ultraviolet irradiation (Beardsley, 1955)	E. W. Nester
B6-806(PS8)	Strain B6-806 lysogenized with bacteriophage PS8 (Chilton et al., 1974a)	"
A277	Virulent transconjugant from *in planta* mating of B6-806 and A136 (NT1 made rif^r, nal^r), contains pTi-B6-806	"
A217	Avirulent derivative of B6-806 isolated after treatment with nitrosoguanidine, contains pAt-B6-806 (Sciaky et al., 1978)	"
B6-Braun	B6 strain originally obtained from A. Braun	"
TR1WS	B6 strain found contaminating *A. radiobacter* strain TR1: TR1 when cloned grew with wrinkled (TR1W) and smooth (TR1S) morphology, TR1W when cloned grew with rough (TR1WR) and smooth (TR1WS) morphology (Farrand, unpublished observation)	"
B6-W	B6 strain originally from J. Kemp	"
B6-M	B6 strain originally frm C. Pootjes	"
B6-S	B6 strain originally from C. Pootjes	"
B6-Heb	B6 strain originally from G. Heberlein	"
B6-Moore	B6 strain originally from L. Moore	"
B6-Schil	B6 strain originally from R. Schilperoort	"
B6-Tourneur	B6 strain originally from Tourneur; has insert in Sma I fragment 2 of Ti-plasmid (Sciaky et al., 1978)	"

TABLE 1. Cont'd.

Strain	Description	Source
B6-Miller	B6 strain originally from A. Miller	A. Matthysse
23308	American Type Culture Collection (ATCC) strain of B6	"
11156	ATCC strain of Braun's B6, smooth morphology	J. Lippincott
11157	ATCC strain of Braun's B6, intermediate morphology	"
11158	ATCC strain of Braun's B6, rough morphology	"
B6	B6 strain, originally from Stonier	"
B6-6	B6 strain, originally from Beardsley	"
B6-Man	B6 strain originally from P. Manigault	"
C58	Agrobacterium strain that can be cured of Ti-plasmid by growth at 37°C, bacteriocin 84 sensitive, nopaline utilizer (Hamilton and Fall, 1971)	E. W. Nester
NT1	A heat cured derivative of C58 resistant to bacteriocin 84, cannot utilize nopaline (Watson et al., 1975)	"
NT1(RP4)	By transformation with RP4	This Thesis
NT1(pTi-B6-Braun)	By transformation with pTi-B6-Braun	"
NT1(pTi-TR1WS)	By transformation with pTi-TR1WS	"
J53(RP4)	Escherichia coli J53 strain (met^-, pro^-, F^-, $\lambda+$); drug resistance plasmid RP4 (Ap/Cb, Km/Nm, Tc)	S. K. Falkow

Buffers. TES buffer was composed of 0.05 M tris(hydroxymethyl)aminomethane-HCl, 0.05 M sodium chloride and 0.005 M ethylenediaminetetraacetic acid (EDTA), adjusted to pH 8.0 by addition of solid NaOH. TE buffer contained 0.05 M Tris-HCl pH 8.0 and 0.02 M EDTA, pH 8.0. Standard saline citrate (1 X SSC) consisted of 0.15 M sodium chloride and 0.015 M trisodium citrate. Phosphate buffer (PB) used in the DNA/DNA hybridization studies was an equimolar mixture of dibasic sodium phosphate and monobasic sodium phosphate. TEN buffer for E. coli transformation contained 0.02 M Tris-HCl pH 8.0, 0.001 M EDTA and 0.02 M sodium chloride.

3-ketolactose test. The 3-ketolactose test, described by Bernaerts and De Ley (1963), is a specific biochemical test for the identification of crown gall bacteria. Strains to be tested were first grown on chalk agar for 2 days at 25-30°C. A loopful of bacterial growth was then transferred to a lactose agar plate and incubated at 28°C for 2 days. Following incubation, the plate was flooded with Benedicts reagent (0.59 M sodium citrate, 0.94 M sodium carbonate and 0.07 M cupric sulfate). A positive reaction is indicated by a yellow ring of Cu_2O appearing around the colonies.

Bacteriophage studies.

Test for lysogeny. Agrobacterium strains were tested for lysogeny by Omega type bacteriophages capable of infecting strain B6-806. Each strain was grown overnight in 5 ml of L-broth. The cells were pelleted by centrifugation and the culture supernatant was filter sterilized by passage through a 0.45 μm (pore size) membrane filter (Millipore

Corp.). Each supernatant was serially diluted in L-broth and the dilutions tested for the presence of bacteriophage by soft agar assay (Adams, 1959) using strain B6-806 as an indicator. One tenth ml of each dilution, 3 drops of the indicator bacterial suspension grown in L-broth to a turbidity of 50 Klett units, red filter, and 3 ml of soft agar were gently mixed and spread on a nutrient (NA) agar plate. After overnight incubation at 30°C each set of plates was examined for plaques. The presence of plaques indicated that the particular strain of bacteria from which the supernatant was derived harbored a phage that was able to propagate on strain B6-806. Controls included the plating of the indicator strain alone and of each supernatant alone.

Test for sensitivity. Each Agrobacterium strain was tested for sensitivity to authentic PS8 bacteriophage. Strain B6-806 (PS8) was the source of authentic PS8 stock phage. A 10 ml L-broth culture of B6-806 (PS8) was grown to late log phase. The cells were pelleted by centrifugation and discarded. The culture supernatant was filtered through a 0.45 μm (pore size) membrane filter and stored at 4°C. Soft agar assays were performed as described above using each Agrobacterium strain as an indicator strain. Each culture was grown to a turbidity of 50 Klett units and a PS8 stock preparation was diluted so each strain was plated with approximately 200 plaques forming units (pfu) of phage. The plates were examined for plaques after overnight incubation at 30°C. The presence of plaques indicated that the particular strain of bacteria was sensitive to PS8.

Octopine utilization. The ability to utilize octopine is a character-

istic coded for by the Ti-plasmid (Bomhoff et al., 1976, see introduction). Each strain was assayed for octopine utilization by testing its ability to grow with octopine as sole source of carbon and nitrogen. A single colony of the strain to be tested was suspended in one ml basal medium supplemented with 500 µg/ml octopine. Cultures were incubated at 29°C and observed for growth at 24 h, 48 h, and 72 h intervals.

Tumorigenicity assays.

Carrot disc assay. The use of carrot slices for detection of crown gall bacteria has been discussed by Ark and Schroth (1958). Fresh carrots were thoroughly washed and soaked for 10 min in a 20% solution of sodium hypochlorite. Each was rinsed with sterile distilled water, dipped in a 95% solution of ethanol and passed through a flame to be surface sterilized. Slices 0.6 cm thick were cut with a sterile knife and placed apical side up in plastic petri dishes. The dishes were lined with moist sterile filter paper to prevent the carrot discs from drying out before the assay was completed. Carrot slices were inoculated with a single strain of bacteria by picking a colony with a toothpick and smearing the cells on the cut surface. One slice in each dish was left uninoculated as a control. After 12 days carrot discs were examined for induction of tumor formation by the bacterial strain tested.

Kalanchoe leaf assay. The Kalanchoe virulence assay has been described by Watson et al. (1975). Healthy Kalanchoe leaves were wounded with sterile wooden applicator sticks. The wound sites were

inoculated by toothpick with colonies of the different bacterial strains. One wound site on each leaf was left uninoculated as a control. After 24 days each leaf was examined for evidence of tumor formation.

Isolation of DNA.

Isolation of plasmid DNA from A. tumefaciens. A modification of the Currier and Nester technique (1976b) was used to isolate plasmid DNA from the Agrobacterium strains. A one liter L-broth culture of cells was grown to a turbidity of 100 Klett units at 29°C with aeration. Cells were harvested by centrifugation at 11,000 x g for 10 min and washed once with AB minimal medium lacking glucose. The cell pellet was resuspended in 200 ml of TE buffer and Pronase B (Calbiochem., predigested by incubation at 37°C for 90 min at a concentration of 5 mg/ml in TE) added to a concentration of 500 µg/ml. After 30 min incubation at 37°C the lysate was adjusted to 1% sodium dodecyl sulfate (SDS) and incubated at 37°C until clear. The lysate was sheared either by vortexing 10 ml aliquots for 2 min at the maximum setting of a Scientific Products Vortex Genie Mixer (Model No. S8223) or by 3 passages through an 18 gauge hypodermic needle. The DNA in the lysate was denatured by dropwise addition of 3 N sodium hydroxide until the pH of the solution was at 12.3 to 12.4. The lysate was stirred for 7 min and the pH was rapidly lowered to 8.4 by addition of 2 M Tris-HCl pH 7.0. The lysate was adjusted to 3% sodium chloride by addition of solid salt and extracted with one volume of redistilled phenol saturated with 3 X SSC. Following centrifugation for 10 min at 11,000 x g,

the aqueous layer was removed and extracted with one volume of chloroform:isoamyl alcohol (24:1, v/v). The mixture was centrifuged for 5 min at 11,000 x g and the aqueous layer was removed and adjusted to 0.3 M sodium acetate. One volume of cold 95% ethanol was added, the solution was mixed well and stored at -20°C overnight. Precipiated DNA was collected by centrifugation (11,000 x g, 30 min, -4°C) and redissolved in TES buffer for purification by cesium chloride-ethidium bromide density equilibrium gradient centrifugation. Each gradient tube contained 7.4 ml DNA dissolved in TES, 0.6 ml ethidium bromide solution (10 mg/ml in TES) and 8 g cesium chloride. The refractive index of the solution was adjusted to 1.3940. The preparation was centrifuged at 85,000 x g (32,000 rpm) for 72 h at 20°C (40 rotor, Beckman L5-65 ultracentrifuge). Bands of DNA at equilibrium were visualized under long wave ultraviolet (UV) illumination. The lower band corresponding to plasmid DNA, was collected in a minimal volume by either using a needle and syringe to puncture the centrifuge tube and withdraw the band from the side, or after aspiration of the upper portion of the gradient, a cut-off pasteur pipet was used to withdraw the band from the top. When required, plasmid DNA was further purified by re-centrifugation in a second cesium chloride-ethidium bromide density equilibrium gradient. A solution was prepared containing 7.4 ml TES, 0.6 ml ethidium bromide stock of 10 mg/ml, 8 g cesium chloride and adjusted to a refractive index of 1.3920. An appropriate amount of this mixture was added to the plasmid DNA (in cesium chloride ethidium bromide) to bring the volume to 10 ml. The refractive index of the new solution was adjusted to 1.3920 and centrifugation carried out as

described above. Following centrifugation and collection, ethidium bromide was removed by extraction with isopropyl alcohol saturated with 20 X SSC. The cesium chloride was removed by dialysis against the appropriate buffer and DNA was stored at 4°C over chloroform.

The concentration of DNA in each plasmid sample was determined by spectrophotometry (Eisinger and Lamola, 1971). The absorbance of a 1:20 dilution of each DNA sample at 260 nm was obtained on a Gilford spectrophotometer. This reading was corrected for light scattering and converted directly to mg DNA/ml.

Isolation of plasmid DNA from Escherichia coli.

Plasmid DNA was isolated from E. coli by a modification of the procedure of Clewell and Helinski, 1969. A one liter L-broth culture of cells was grown to a turbidity of 100 Klett units at 37°C with aeration. Cells were harvested by centrifugation at 11,000 x g for 10 min and resuspended in 33 ml of cold solution A (25% sucrose in 0.05 M Tris-HCl pH 8.0). After addition of 6.6 ml of a solution of lysozyme (5 mg/ml in 0.25 M Tris-HCl, pH 8.0) the cell suspension was incubated on ice for 5 min. Next 11.4 ml of 0.25 M EDTA was added and incubation on ice continued for an additional 5 min. Lysis was brought about by addition of 52 ml of solution B (1% Brij 58, 0.4% sodium deoxycholate, 0.625 M EDTA, 0.05 M Tris-HCl, pH 8.0). The suspension was incubated at room temperature until lysis was complete (5-10 min). the lysate was centrifuged at 4°C for 25 min at 43,000 x g, the supernatant collected, the volume doubled with distilled water, and extracted once with an equal volume of redistilled phenol saturated with 0.05

M Tris-HCl, pH 8.0. After centrifugation for 10 min at 11,000 x g the aqueous layer was collected and extracted once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). Following recentrifugation (11,000 x g, 5 min) the aqueous layer was adjusted to 0.3 M sodium acetate and the DNA precipitated, collected and redissolved as described above. The plasmid DNA was purified by cesium chloride-ethidium bromide equilibrium gradient centrifugation as described for A. tumefaciens.

The modified Currier-Nester technique could also be used to isolate plasmid from E. coli. Cells were grown, harvested and washed as described previously. The cell pellet was resuspended in 80 ml 0.05 M Tris-HCl, pH 8.0. After addition of 6.6 ml of the lysozyme solution, the cell suspension was incubated for 5 min on ice. Next 28 ml of 0.25 M EDTA was added and the mixture again incubated for 5 min on ice. Finally, the lysate was adjusted to 1% SDS and incubated at room temperature until clear. Once lysed, the preparation from E. coli was treated exactly as indicated above for lysates of A. tumefaciens.

Isolation of total bacterial DNA.

Total cellular DNA was isolated from all available B6 strains using a modification of the Marmur procedure (1961). A one liter L-broth culture of each strain was grown to late log phase. The cells were harvested, washed once in TE buffer and resuspended in one tenth the original culture volume of TE containing 500 µg/ml self-digested Pronase B. After 30 min at 37°C, SDS was added to the cell suspension to a final concentration of 1% and incubation was continued at 37°C

until lysis was complete. The lysate was extracted with an equal volume of phenol saturated with 0.1 M Tris-HCl, pH 8.0. The phases were separated by centrifugation and the aqueous layer was collected and adjusted to 1 X SSC. This solution was overlayered with two volumes of cold 95% ethanol and the DNA spooled out at the interface on a glass rod. Excess ethanol was pressed out and the DNA was dissolved in 10 ml 0.1 X SSC. Once dissolved the solution was adjusted to 1 X SSC and the phenol extraction and ethanol precipitation repeated. The solution of DNA was extracted with an equal volume of chloroform:isoamyl alcohol (24:1, v/v), overlayered with ethanol, spooled out and redissolved in 10 ml 0.1 X SSC. The solution was adjusted to 2 X SSC and one one-hundredth volume of a stock mixture of RNase A (20 mg/ml) and RNase T1 (600 units/ml dissolved in 0.15 M sodium chloride, 0.01 M sodium acetate, pH 5.2) added. Incubation was carried out at 37°C for 30 min and the solution was extracted with an equal volume of phenol saturated with 0.1 M Tris-HCl, pH 8.0. Following centrifugation, the aqueous phase was extracted with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The mixture was centrifuged, the aqueous layer removed, and overlayered with ethanol. The precipitated DNA was spooled out and redissolved in a minimal volume of 0.1 X SSC. The purified DNA was dialyzed against 0.1 X SSC and stored over chloroform.

Isolation of radiolabeled plasmid DNA.

Labeling conditions. A 10 ml preculture of the strain to be labeled was grown overnight in AB minimal medium supplemented with 0.5% glucose and 0.05% vitamin free casamino acids (Difco). The

preculture was used to inoculate 300 ml of this medium to a turbidity of 10 Klett units. Tritiated thymidine (specific activity 56.9 Ci/mmol, New England Nuclear) was added in graded aliquots at every culture mass doubling. When a total of 2 mCi had been added the culture mass was allowed to double once more before harvesting at a final turbidity of about 60 Klett units.

Isolation of plasmid. Plasmid DNA was isolated and prepared for cesium chloride ethidium bromide equilibrium gradient centrifugation as described above.

Fractionation of cesium chloride gradient and determination of radioactivity. Cesium chloride gradients containing radiolabeled DNA were fractionated as follows. The centrifuge tube was punctured from the bottom and 15 drop fractions collected into 12 x 75 mm disposable test tubes. Ten µl aliquots of each fraction were spotted onto numbered Whatman filter paper discs. Radiolabeled nucleic acids were precipitated onto the filters by two washes with 5% trichloroacetic acid (TCA, Mallinckrodt, Inc.). The TCA-treated filters were rinsed twice in cold 95% ethanol and dried. Each filter was placed in a scintillation vial containing 7 ml scintillation cocktail (Liquifluor, New England Nuclear, diluted to a final concentration of 4 g PPO and 50 mg POPOP per liter of toluene) and radioactivity from each fraction determined by scintillation spectrometry (Beckman LS 8000 Liquid Scintillation Spectrometer). The fractions containing radiolabeled plasmid DNA were pooled and purified on a second cesium chloride ethidium bromide gradient. The second gradient was fractionated and assayed in the same manner as the first and the fractions containing

the labeled plasmid DNA pooled. The ethidium bromide was removed by extraction with isopropyl alcohol as described above and the DNA was dialyzed against 0.15 M PB and stored at 4°C.

Electron microscopy.

Spreading of DNA. Plasmid DNA, isolated as described above, was prepared for electron microscopy by the Kleinschmidt technique (Kleinschmidt and Zahn, 1959; Kleinschmidt, 1968; Lang and Mitani, 1970) as described by S. Falkow (personal communication). A plastic petri dish was filled with hypophase (0.25 M ammonium acetate, pH 8.4, Mallinckrodt, Inc.) purified by filtration through a 0.45 µm (pore size) nitrocellulose membrane (Millipore Inc.). A wet acid-cleaned (overnight soak in 45 g potassium dichromate dissolved in 1 l sulfuric acid), glass slide was rinsed in hypophase, placed against the side of the dish and allowed to dry. The spreading solution was prepared by adding together 50 µl 1 M ammonium acetate pH 8.4, 10 µl cytochrome c (Sigma, 1 mg/ml in double distilled water) and 40 µl DNA in TE buffer. One drop of this solution was spotted on the dry slide just above the hypophase meniscus and allowed to spread over the hypophase for 60 seconds. Parlodian coated copper grids were prepared as follows. The grids, resting on a wire screen were placed in a large funnel which had been filled with double distilled water. Parlodian (previously baked at 90°C, 24 h) was prepared as a 3.5% solution in dry isoamyl acetate. A drop of parlodian solution was placed on the surface of the water and allowed to spread. The parlodian film was lowered onto the grids by slowly draining the water from the funnel

and the coated grids were dried and baked at 60°C oven for 15 min before use. The spread DNA was picked up by touching a coated grid, film side down, to the hypophase surface one grid width away from the slide. The wet grid was immediately dipped into uranyl acetate stain solution (80 µl of 0.05 M uranyl acetate in 0.05 M HCl in 95% ethanol added to 10 ml of 90% ethanol) and held there for 30 seconds. The stained grid was rinsed in 2-methyl butane (Aldrich Chemical Co., Inc.) for 10 seconds before air drying. Grids were visualized directly or rotary shadowed with carbon-platinum or platinum-palladium-tungsten (2 mm of 8 mil wire, 80% platinum-20% palladium; Aberman and Salpeter, 1974). Electron photomicrographs were taken of random well-laid out open circular molecules. Photomicrographs were also taken of carbon coated diffraction gratings (Ladd #1002, 2160 lines/mm; Zaenen et al., 1974).

Plasmid size estimation. Size estimates of plasmid DNA was determined by contour-length measurements of projected plasmid images (Lang et al., 1967). Plasmid DNA images were projected onto newsprint and their outlines traced. Negatives of diffraction gratings were also projected and the distance between two lines of the grating was traced. As each negative was removed from the enlarger, a transparent centimeter rule was placed in the enlarger and the length of one centimeter was traced without refocusing. Knowing the final magnification of the plasmid image, the linear measurement obtained with a Dietzgen map tracer was converted to true length in microns. A sample calculation is given here.

Electron microscope magnification:

-Diffraction grating (negative #202) at tap 3 projected distance between two lines was 23 mm

-a 1 cm line was enlarged 13.6 times when projected onto newsprint paper

$$\frac{2159 \text{ intervals}}{\text{mm}} \times \frac{23 \text{ mm}}{\text{interval}} \times \frac{1}{13.6} = 3651.2$$

-a specimen taken at tap 3 was magnified 3651.2 times by electron microscopy

Length of the plasmid in microns:

-a plasmid (negative #71) at tap 3 - map tracer reading was 318 cm, this is the contour length measurement of an image projected on newsprint

-a 1 cm line was enlarged 14.2 times when projected onto newsprint paper

$$318 \text{ cm} \times \frac{1}{14.2} \times \frac{1}{3651.2} = 0.0061334 \text{ cm or } 61.334 \text{ μm}$$

-the length of the plasmid is 61.334 μm

Molecular weight of the plasmid:

-molecular weight is calculated using the conversion factor:

$1 \text{ μm} = 2.07 \times 10^6 \text{ dal}$ (Clowes, 1972; Lang, 1970; Meyers et al., 1976)

$61.3 \text{ μm} \times 2.07 \times 10^6 \text{ dal/μm} = 126 \times 10^6 \text{ dal}$

Bacterial transformation.

Transformation of A. tumefaciens. Strains of A. tumefaciens were transformed as described by Holsters et al. (1978). The recipient bacteria were grown in 100 ml of L-broth at 28°C with aeration to a turbidity of 100 Klett units. The cells were washed once with 0.01 M Tris-HCl pH 7.5 and concentrated 100 X by resuspension of the sedimented cells in one ml of L-broth. Two tenths ml of concentrated cells

(about 10^{11} colony forming units or cfu/ml) were mixed with 0.1 ml of purified plasmid DNA (20-137 µg/ml in 1 mM Tris pH 7.5, 0.25 mM EDTA pH 8.0) and immediately frozen at -80°C in a dry ice ethanol bath and kept at this temperature for 5 min. The mixture was thawed at 37°C, kept for 25 min at this temperature and then diluted 1:5 in fresh L-broth and incubated with aeration at 28°C. At various timed intervals 0.1 ml samples of the mixture were plated on selective media. When recipient cells were transformed with RP4 plasmid DNA, transformants were selected on NA plates containing 20 µg kanamycin/ml. When transformations were performed with Agrobacterium Ti-plasmid DNA the transformants were selected on AB minimal agar containing 500 µg/ml of octopine as sole carbon source. Controls in each transformation included plating of recipient bacteria on selective medium and plating of DNA on nutrient agar.

Transformation of E. coli. Escherichia coli was transformed with plasmid DNA as described by Lederberg and Cohen (1974). A 50 ml L-broth culture was inoculated with 0.5 ml of an overnight preculture of the receipient. When the culture had reached an optical density of 0.6 (measured at 600 nm, Bausch and Lomb Spectronic 20) the cells were chilled on ice and then harvested by centrifugation (10 min at 12,000 x g, 4°C in precooled sterile centrifuge bottles). Cells were washed once with 50 ml cold sterile 0.1 M $MgCl_2$, resuspended in 25 ml of cold sterile 0.1 M $MgCl_2$ and chilled for 20 min on ice. Cells were harvested and resuspended in 2.5 ml cold sterile 0.1 M $CaCl_2$ in a sterile test tube. One tenth ml of purified plasmid DNA was mixed with 0.2 ml of the "competent" recipient bacteria and chilled on ice for 30 min. The

mixture was warmed at 42°C for 2 min for uptake of DNA and placed on ice again until chilled. The transformation mixture was diluted 1:100 in prewarmed L-broth and incubated at 37°C with aeration for expression. At timed intervals 0.1 ml aliquots of the mixture were removed and plated on selective medium. A sample of the recipient bacteria was similarly treated in the absence of purified plasmid DNA and plated as a control.

Preparation of complementary RNA.

Synthesis. Complementary RNA (cRNA) was synthesized essentially as described by Ketner and Kelly (1976). The one ml reaction mixture contained 10 mM $MgCl_2$, 140 mM KCl, 33 mM Tris-HCl pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 10% v/v glycerol, 124 µg/ml bovine serum albumin and 0.5 mM ribonucleotide triphosphates including ^{3}H-ATP (49.6 Ci/mmol), ^{3}H-UTP (27 Ci/mmol), ^{3}H-GTP (16.7 Ci/mmol) plus 20 µg DNA. Isotopes were obtained from ICN Pharmaceuticals, Inc. Fifteen units of RNA polymerase (Boehringer-Mannheim) was added to initiate the reaction and the mixture incubated at 37°C for 5 h. Experiments which established these conditions are described in the results.

Synthesis of RNA was halted by addition of deoxyribonuclease I (DPFF, RNase free, Worthington Biochemical Corp.) to a final concentration of 20 µg/ml and incubation of the mixture at 37°C for 30 min. The cRNA was extracted twice with an equal volume of phenol saturated with 0.1 M Tris-HCl, pH 8.0 and twice with an equal volume of diethyl ether. Unincorporated ribonucleotide triphosphates were removed by chromatography (Wensink et al., 1974) on Sephadex G-75 (Sigma). The

gel bed (1.5 x 24 cm) was equilibrated with 0.1 X SSC. The buffer meniscus was lowered to 1 mm above the gel bed before the cRNA was layered onto the column. When the sample meniscus had also been lowered to 1 mm above the gel bed eight ml 0.1 X SSC was carefully added to the column and the buffer reservoir connected. Fifteen drop fractions were collected from the column. Two 10 µl aliquots of each fraction were spotted onto filter paper. One set of filters was dried and radioactivity from each fraction was determined as in "Isolation of radiolabeled plasmid". The second set of filters was washed with 5% TCA and rinsed with ethanol before radioactivity from each fraction was determined. Fractions containing TCA precipitable radioactivity (labeled cRNA) were pooled.

The product of the cRNA reaction was analyzed on sucrose gradients. Seventy-five µl of a sample was layered onto a 5-20% wt% neutral sucrose gradient (0.55 M NaCl, 0.05 M Tris, 0.005 M EDTA, pH 8.0) and centrifuged at 125,000 x g for 12 h at 20°C (SW 50.1 rotor, Beckman L5-65 ultracentrifuge). The centrifuge tubes were punctured from the bottom and eight drop fractions collected into 12 x 75 mm disposable test tubes. A 10 µl aliquot of each fraction was spotted onto filter paper, washed with 5% TCA and radioactivity from each fraction determined as described under "Isolation of radiolabeled plasmid DNA".

Quantitation. The amount of RNA present in the pooled volume was determined by the orcinol reaction as described by Schneider (1957). One and one half ml of orcinol reagent (0.5 g orcinol, Eastman Kodak Co., and 0.25 g ferric chloride in 50 ml of concentra-

ted HCl) was combined with an equal volume of RNA sample in 0.1 X SSC, heated in a boiling water bath for 20 min, cooled and the absorbance at 660 nm determined in a Bausch and Lomb Spectronic 70. A standard curve was prepared from the absorbance readings of a series of samples containing known concentrations of yeast RNA (Sigma). The absorbance reading of an aliquot of cRNA product was compared with the standard curve to determine the cRNA concentration.

In situ colony hybridization.

In situ lysis. Various Agrobacterium strains were streaked onto nitrocellulose filters (Schleicher and Schuell; boiled three times in distilled water, autoclaved in distilled water), placed on NA plates and allowed to grow for 36 h. The method of in situ lysis described by Grunstein and Hogness (1975) was modified for Agrobacterium. The filters were carefully moistened from below (to avoid smearing of the colonies) with lysis buffer (1% SDS, 500 µg/ml self-digested Pronase B in TE buffer), sealed in a petri dish and incubated at 37°C for 90 min. After lysis, filters were moistened with 0.5 N NaOH for 20 min at room temperature to denature nucleic acids. The pH was adjusted to neutrality by two treatments with 1 M Tris-HCl pH 7.5; and the filters were treated with 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5 for 5 min to dissociate protein from the nucleic acid. Filters treated in this fashion were placed on a Millipore filtration apparatus and a vacuum applied until the colonial residues were dry in appearance. After this fixation, there was less danger of movement of material from the colonial site and the remainder of the solutions. were layered gently on the

top of the filters.

The filters were moistened with a solution of Proteinase K (Beckman, 2 mg/ml in 1 X SSC) for 15 min, washed once with 95% ethanol and five times with chloroform. Debris was removed by dipping the filter in 0.3 M NaCl. The filters were partially dried at room temperature and baked at 80°C *in vacuo* for 2 h.

Colony DNA:cRNA Hybridization. The filters, prepared as described above, were moistened with a solution of 5 X SSC, 50% foramide (Matheson, Coleman and Bell; McConaughy et al., 1969), and 25% of the solution volume was composed of the ^{3}H-cRNA stock preparation. Two hundred µl of hybridization mixture was applied to the top of each filter. The filters were then covered with mineral oil and incubated for 16 h at 37°C (Gillespie and Spiegelman, 1965; Grunstein and Hogness, 1975). After incubation all filters were washed three times for 10 min each in chloroform. This was followed by successive 10 min washes in 6 X SSC and 2 X SSC containing 20 µg/ml pancreatic RNase. The filters were then baked for 30 min at 80°C *in vacuo*.

The extent of hybridization was qualitatively assessed by fluorography (autoradiography, Randerath, 1970). A 7% solution of 2, 5-diphenyloxazole (PPO) in ether was used to enhance tritium detection. Forty µl was applied per square centimeter of filter. When the filter had dried, it was placed in direct contact with Kodak X Omat R-5 x-ray film, and stored at -82°C for 12 days.

Alternatively, areas of the filters containing the colonial residues were cut apart and each filter section placed into 5 ml of Liquifluor:toluene scintillation cocktail. Radioactivity was determined by

scintillation spectrometry as previously described.

Solution hybridization.

Preparation of DNA. Samples of unlabeled, total bacterial DNA were adjusted to 250 µg/ml in 0.1 X SSC and sheared in a French pressure cell at 12,000 pounds per square inch (psi). The sheared DNA was dialyzed exhaustively against distilled water to remove all salts. Aliquots of these DNA preparations were evaporated to dryness in 12 x 75 mm disposable test tubes at 37°C under a stream of air.

Hybridization reactions. Routinely the dried DNA samples were redissolved to a final concentration of one mg unlabeled DNA/ml, in 65 µl of the radiolabeled plasmid DNA in 0.15 M PB. This DNA mixture was drawn up in six 10 µl capillary tubes and the tube ends were flame sealed. Each set of capillaries was denatured by boiling at 105°C for 7 min in salt water. The reaction tubes were immediately transferred to a water bath and the renaturation allowed to proceed at 67°C. Capillaries were removed from the water bath at timed intervals and chilled in ice water to stop the renaturation.

Duplex assay. Contents of the capillaries were blown into one ml aliquots of cold 0.15 M PB and loaded onto columns of hydroxyapatite (Kohne and Britten, 1975) at 67°C. The columns were washed twice with 1 ml 0.15 M PB. The flow through and wash volumes were collected directly in scintillation vials. The columns were then washed with 2 ml of 0.3 M PB and the wash volume collected in a second set of scintillation vials. All samples were adjusted to a volume of 4 ml and a salt concentration of 0.15 M PB. Fifteen ml of scintillation cocktail

were added to each vial. The cocktail was prepared by mixing 200 ml Fluoralloy TLX (Beckman), 1000 ml Triton X 100 and 1800 ml toluene. The amount of radioactivity present in both the single stranded fraction and the double stranded fraction was determined for each timed sample.

RESULTS

Electron microscopy. Initial evaluation of group B6 A. tumefaciens plasmids began with the determination of size measurement of plasmid DNA isolated from the strain B6-806, B6-Braun, TR1WS and A217. An electronphotomicrograph of one of the plasmid molecules measured is presented in Figure 1. The average contour length and molecular mass of the plasmid molecules measured are listed in Table 2. All mass and length measurements represent the average obtained for at least 20 independent molecules. Forty plasmid molecules from B6-806 were measured since strain B6-806 is a prototype strain and harbors two plasmids. The average molecular mass of the 40 B6-806 plasmid molecules was 126 x 10^6 dal. The average molecular mass of the B6-Braun plasmid was also 126 x 10^6 dal and that of strain TR1WS was 121 x 10^6 dal. The molecular mass of the strain A217 plasmid at 114 x 10^6 dal was slightly smaller than these. Strain B6-Braun and strain TR1WS plasmids had not been previously measured. To determine if realistic plasmid size estimates had been obtained by these manipulations a plasmid of known size was isolated and measured. Measurements of RP4 DNA were obtained by the same method that was used for the Agrobacterium plasmids (Table 2). The molecular mass of RP4 was calculated to be 38 x 10^6 dal, a value consistent with that in the literature (Stanish and Ortiz, 1976).

The contour length measurements of strain B6-Braun and strain TR1WS plasmid each fell into a single size class which overlapped the B6-806 plasmid size distribution (Figure 2). The strain A217 plasmid was also of a single size class, but was slightly smaller than the

Figure 1. Electronphotomicrograph of pAt-B6-806. Plasmid DNA was isolated, purified and prepared for electron microscopy, as described in Materials and Methods.

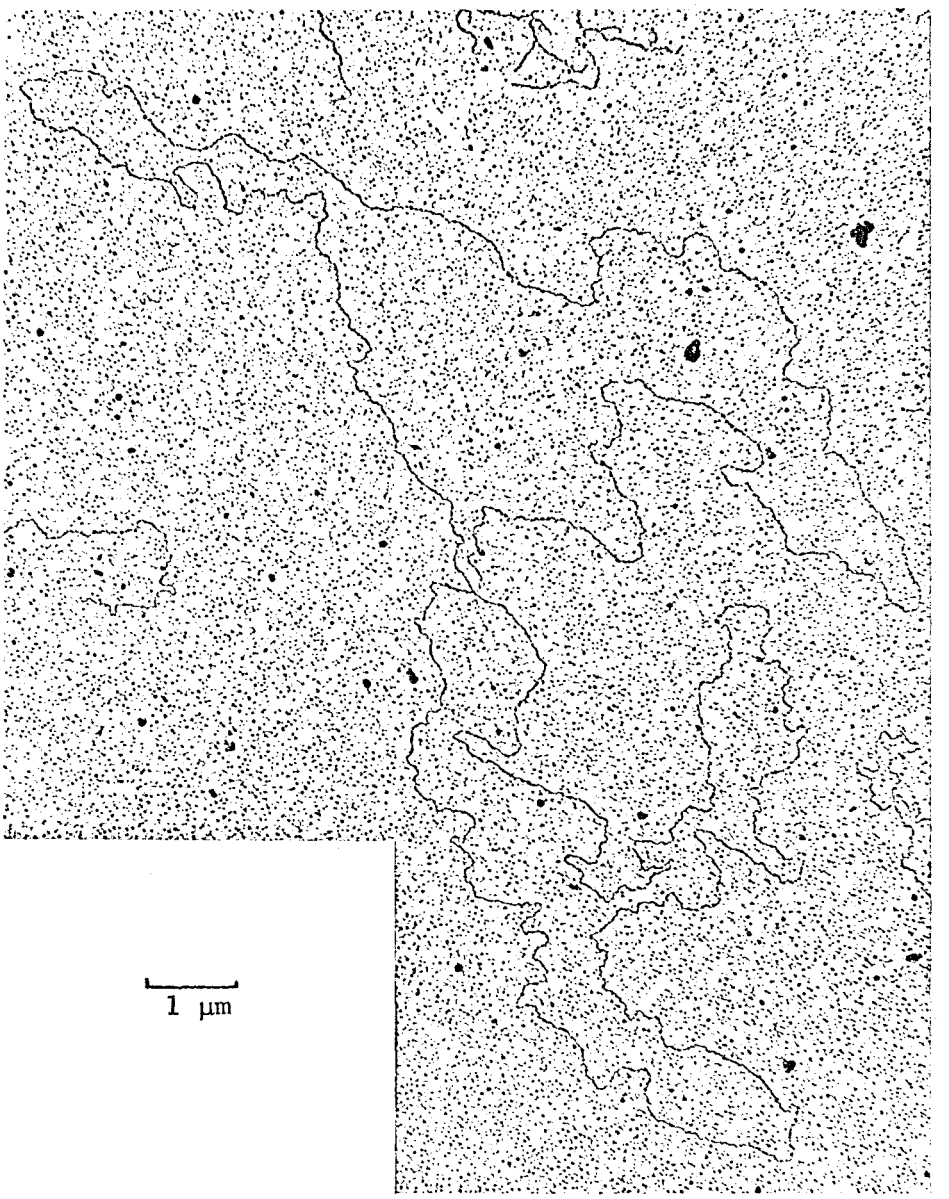

1 µm

TABLE 2

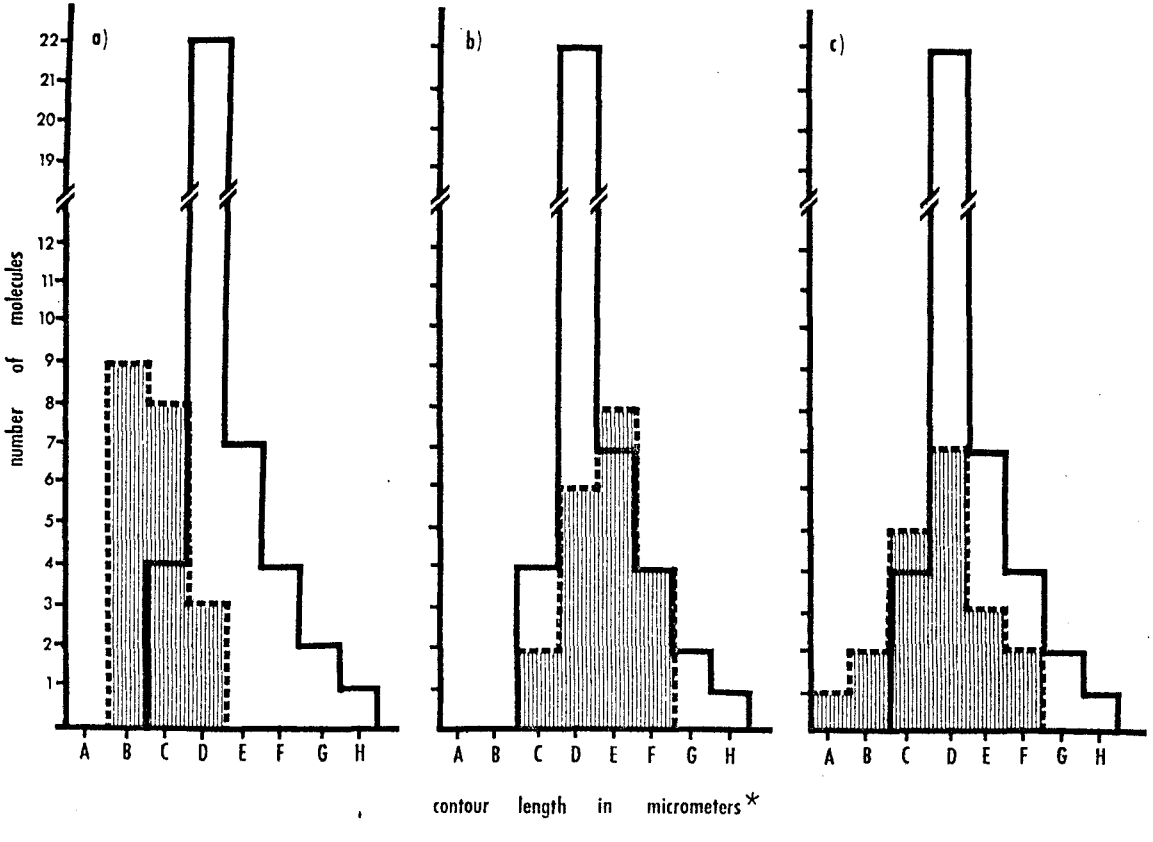
Average Length and Computed Molecular Mass of Plasmids Isolated

Source of Plasmid DNA	n	Average Length (μm)	Std. Error Length (μm)	Molecular Mass [a] (dal)	Std. Error Molecular Mass (Mdal)
B6-806	40	60.6	±0.54	126×10^6	± 1.1
A217	20	55.0	±0.36	114×10^6	±0.74
TR1WS	20	58.6	±0.82	121×10^6	±1.7
B6-Braun	20	61.1	±0.6	126×10^6	±1.2
J53(RP4)	69	18.3	±0.09	38×10^6	±0.18

a Molecular mass was calculated from the contour length measurements of the plasmids, using a conversion factor of 1 μm = 2.07×10^6 dal. (Clowes, 1972; Lang, 1970; Meyers et al., 1976).

Figure 2. Nomogram of Plasmid Contour Length Measurements.

A comparison of size distribution of contour lengths of B6-806 (solid line) with: a. A217 (dashed line); b. B6-Braun (dashed line) and c. TR1WS (dashed line). * Letters on X-axis represent plasmid contour length measurements in increments of 3 μm ranging from A (49-51 μm) to H (70-72 μm).


a)
b)
c)
number of molecules
22
21
20
19
12
11
10
9
8
7
6
5
4
3
2
1
A B C D E F G H
contour length in micrometers *

plasmids from the virulent strain (Figure 2). It was evident from Figure 2 that the size distribution of strain A217 plasmid and strain B6-Braun plasmid when taken together did not cover the same range as the size distribution of the B6-806 plasmid complement. These results neither support not discredit the hypothesis that host specificity markers may be located on the plasmids or the idea that the two B6-806 plasmids may have been segregated and strain B6-Braun harbored only the Ti-plasmid and strain A217 harbored only the cryptic plasmid.

Transformation of B6-Braun and TR1WS

One method to test the hypothesis that host specificity markers are located on the B6 plasmids would be to transfer pAt-B6-806 into strains B6-Braun and TR1WS and to determine the host range of the strains after they received the cryptic plasmid. Plasmid pAt-B6-806 carries no known marker making it impossible to select directly for a B6-Braun (pAt-B6-806) transconjugant or transformant. Kretschmer et al. (1975) have described a cotransformation which relies on the use of a selectable plasmid as an indicator or probe to identify cells that have acquired a second plasmid that is otherwise nonselectable. These workers have shown that when the molar ratio of selectable to nonselectable plasmids was 10^{-3} or less, greater than half of the selected transformants also acquired the unselected plasmid.

It was not certain that strains of the Agrobacterium B6 group were transformable. In order to test for this ability, attempts were made to transform strains B6-Braun and TR1WS with RP4 which carried an easily selectable trait, kanamycin resistance.

The kinetics of plasmid expression was first studied. Strains were transformed as described by Holsters et al. (1978), and after varying timed intervals for expression, aliquots of the transformation mixture were plated on selective medium. The number of kanamycin resistant transformants isolated per ml of transformation mixture was low at all timed platings (Table 3, columns 1, 2). After 7 h incubation for expression, the frequency of transformation was 100 transformants per 10^{11} cfu or 1×10^{-9} for B6-Braun(RP4) transformants and 70 transformants per 10^{11} cfu or 7×10^{-10} for TR1WS(RP4) transformants and by 20-24 h after initiation of plasmid uptake the frequency of transformation was even lower. The experiment was repeated several times and RP4 was dialyzed against different buffer systems, but the number of transformants isolated per ml could not be increased (data not shown). These results seemed to indicate that these two B6 strains were pooly transformable. It may be possible to transform strain B6-Braun and strain TR1WS with pAt-B6-806. However, it is probable that the number of transformants isolated would be low as they were with RP4. Given these low frequencies, it is doubtful that the strain B6-Braun(pAt-B6-806) or TR1WS(pAt-B6-806) transformant would be detected even after co-transformation.

Not all A. tumefaciens strains were as poorly transformable as strains B6-Braun and TR1WS appear to be. An alternate approach was to sequentially introduce by transformation both pTi-B6-806 and pAt-B6-806 into another strain of Agrobacterium that had been shown to be transformable. If host specificity markers are located on the plasmids, it is the plasmid content of a strain that is important, not the A. tume-

faciens recipient strain used.

Transformation of NT1. A plasmid-free avirulent derivative of strain C58, NT1 (Watson et al., 1975), was successfully transformed with RP4 (Table 3) under conditions previously described. After 5 h of expression, the frequency of transformation was 3×10^{-8} for NT1(RP4) transformants, more than 90 times greater than the number obtained of B6-Braun(RP4) and 30 times greater than the number of TR1WS(RP4) transformants isolated at that time. The number of transformants per ml was observed to decrease after 20-24 h incubation.

These experiments demonstrated that strain NT1 could be transformed with RP4. However, the Ti-plasmids are more than three times larger than RP4. When NT1 was transformed with Ti-plasmid from strain B6-Braun or strain TR1WS, NT1(pTi-B6-Braun) and NT1(pTi-TR1WS) transformants were selected for by their ability to utilize octopine as sole source of C and N. As described in the Introduction, this trait is coded for by the Ti-plasmid (Bomhoff et al., 1976; Kerr, 1975). The number of NT1(pTi-B6-Braun) transformants isolated was greatest after allowing 2-3 h for expression of the selected trait. The frequency of transformation was 4×10^{-10} for NT1(pTi-TR1WS) after 2-3 incubation and was not significantly changed with time.

To determine if these strains were still transformable, NT1(pTi-B6-Braun) and NT1(pTi-TR1WS) were each transformed with RP4 (Table 3). Each of these recipients was found to be transformable at a low level, 1×10^{-10}, but still higher than strain B6-Braun or strain TR1WS.

Several preliminary experiments were necessary before attempting

TABLE 3

Comparison of Transformability of Agrobacterium Strains

Recipient Bacterial Strain	Plasmid DNA Source	Number of Transformants[a]				
		Expression Time				
		0 h	2 h	4 h	7 h	23 h
B6-Braun	RP4	2.2	2.2	4.4	11	1.1
TR1WS	RP4	7.7	5.5	12	7.7	2.2
NT1	RP4	0	250	357	nd[b]	138
NT1	pTi-B6-Braun	476	670	553	218	nd
NT1	pTi-TR1WS	13	6.7	27	6.7	6.7
NT1(pTi-B6-Braun)	RP4	0	1.1	3.3	5.5	4.4
NT1(pTi-TR1WS)	RP4	0	14	22	nd	16

a numbers given are transformants per ml per μg transforming DNA based on the average of three plate counts per time point

b not determined

the cotransformation of NT1(pTi-B6-Braun) or NT1(pTi-TR1WS) with pAt-B6-806. Conditions that had to be defined included the optimum time required for expression before plating the transformation mixture on selective medium; the concentration of RP4 DNA necessary for isolation of approximately 1000 transformants per ml a frequency of 1×10^{-8}, and finally, the effect of an excess of unrelated DNA on the transformation frequency with RP4.

The RP4 stock solution (68 µg RP4 DNA per ml) was serially diluted and transformation of NT1 performed with each DNA dilution. All other experimental conditions were as described above and aliquots of the transformation mixture were plated after timed intervals. The optimum time required for expression of RP4-coded kanamycin resistance was observed to be 5-7 h (Table 4). The quantity of RP4 necessary for isolation of 500-1000 transformants per ml, was 0.8 µg DNA per 0.3 ml transformation mixture (Table 4).

The effect of an excess of unrelated DNA on the transformation of NT1 with RP4 was determined by the addition of excess heterologous E. coli strain J53 total DNA to the transformation mixtures. Duplicate transformation experiments were performed at three concentrations of RP4, 1.7 µg, 0.8 µg and 0.4 µg per transformation mixture. One set of NT1 transformation mixtures received only RP4 DNA. The second set of transformation mixtures received a combination of RP4 and a great excess of E. coli DNA preparation (150 µg). In Table 5, it is evident that the presence of an excess of unrelated DNA did interfere with the efficiency of RP4 uptake, but did not completely inhibit the transformation event. The frequency of transformation was 2×10^{-9} when strain J53 was present

TABLE 4

Determination of Optimum Length of Incubation and Optimum DNA Concentration Used for Transformation of NT1

Incubation Time	Number of Transformants[a]							
	μg RP4 DNA Added							
	6.8	3.4	1.7	0.8	0.4	0.2	0.1	0.05
0 h	30	20	0	0	0	0	0	0
3 h	1110	2000	310	370	100	60	20	10
5 h	1620	2600	390	660	150	100	60	50
7 h	1930	2710	530	600	320	130	30	40
19 h	2370	2070	300	390	30	70	20	40

a numbers given are Kanamycin resistant transformants per ml based on the average of three plate counts per time point

TABLE 5

Effect of Excess Unrelated DNA on Transformation of NT1 with RP4

Transforming DNA per Transformation Mixture		Number of Transformants[a] Incubation Time	
RP4 DNA	J53 DNA	3 h	7 h
1.7 μg	--	1500	2250
0.8 μg	--	580	800
0.4 μg	--	600	750
1.7 μg	150 μg	70	200
0.8 μg	150 μg	20	70
0.4 μg	150 μg	10	40

a numbers given are kanamycin resistant transformants per ml based on the average of three plate counts per time point

compared to a frequency of 2×10^{-8} when the transformation was performed with RP4 DNA alone. When unrelated DNA was present, the greatest number of transformants was obtained when the transformation mixture contained 1.7 μg RP4 DNA (roughly a 1:100 proportion of RP4 to unrelated DNA).

The cotransformation of NT1(pTi-B6-Braun) was performed using a RP4 to pAt-B6-806 DNA concentration ratio of 1:100, corresponding to a genome ratio of 1:33. The transformation mixture was plated for isolation on NA plates containing kanamycin to select RP4-containing recipients after a 7 h incubation for expression. Under these conditions the frequency of transformation was 9×10^{-10} and a total of 762 NT1(pTi-B6-Braun, RP4) transformants were isolated and retained for further study. When the cotransformation was repeated with NT1(pTi-TR1WS) the frequency of transformation was 1×10^{-10} and a total of 95 NT1(pTi-TR1WS, RP4) transformants were isolated for further work. These transformants were to be tested for the presence of the unselected pAt-B6-806.

Identification of recipients of pAt-B6-806. Each of the NT1(pTi-B6-Braun, RP4) and NT1(pTi-TR1WS, RP4) transformants described above was to be individually screened for the presence of pAt-B6-806 until the cotransformed colonies were identified. The rare cotransformant receiving pAt-B6-806 in addition to the selectable RP4 DNA could then be used in a host range study to determine if host specificity markers were carried by plasmids. All transformants selected in the cotransformation were to be screened for pAt-B6-806 plasmid by using an *in situ* colony hybridization technique (Grunstein and Hogness, 1975). In this tech-

nique bacterial colonies are grown on nitrocellulose filters placed on the surface of NA plates. These colonies are lysed on the filters and their DNA is fixed in place and denatured. A solution of radiolabeled RNA complementary to pAt-B6-806 is placed on the filters and the copy RNA (cRNA) allowed to hybridize with any homologous DNA sequences. Any single stranded non-hybridized cRNA is removed from the filters by RNase digestion. Colonies that contain DNA homologous to pAt-B6-806 cRNA can then be identified by autoradiography. Since many colonies can be grown on a single filter, this technique allows a larger number of colonies to be screened simultaneously for the presence of specific nucleotide sequences.

Radiolabeled cRNA made from a pAt-B6-806 template was synthesized and purified for use in the in situ hybridization. The following experiments were all conducted with RP4 template to be sure that the in vitro system worked and then repeated with pAt-B6-806 template. This precaution was taken so any difficulties encountered could be attributed to the DNA template used, not to a deficiency of the system.

Synthesis of copy RNA (cRNA). The cRNA was synthesized from an RP4 or a pAt-B6-806 template using the technique described by Ketner and Kelly (1976). In order to determine the optimum concentration of RNA polymerase, a one ml reaction mixture was split into four aliquots of 0.2 ml. Synthesis of cRNA in these mini reactions was initiated by addition of 3 units, 6 units or 15 units of E. coli RNA polymerase (Boehringer-Mannheim, specific activity: 800 units/mg protein). All reaction tubes were capped and incubated in a 37°C waterbath. Each

reaction mixture was sampled periodically after the addition of enzyme by spotting 5 µl of the reaction mixture on numbered Whatman filter paper discs. The filter discs were washed three times in 5% TCA and rinsed 3 times in cold ethanol. Radioactivity on each disc was determined by liquid scintillation spectrometry as described in Materials and Methods. The kinetics of the three RNA polymerase-initiated reaction mixtures were similar (Figure 3). In addition, the radioactive counts incorporated into cRNA product were not proportional to the amount of enzyme present. After 24 h the percent incorporation ranged from 71-80 percent for the three reactions. If the reactions were allowed to continue for longer than two hours (data not shown) there was no advantage to using a large amount of enzyme. The final percent incorporation of radioactivity was in each case over 70 percent. All subsequent cRNA reaction mixtures were initiated with 15 units of RNA polymerase per one ml reaction mixture.

All cRNA made was purified by Sephadex G-75 column chromatography prior to use in the in situ hybridization. Figure 4 showed that G-75 chromatography effectively removed the TCA soluble radioactivity from the cRNA. The elution profile of RP4 cRNA excluded from the G-75 column (Figure 4) shows the cRNA eluted from the column in fractions 13-18. The cRNA made from an RP4 template had a peak fraction count of 14,000 cpm (Figure 4) in a 10 µl sample. The elution profile of pAt-B6-806 cRNA purified on a sephadex column was also graphed (not shown). The pAt-B6-806 cRNA had a peak fraction count of 1,200 cpm in a 10 µl sample. This indicated the pAt-B6-806 cRNA was either present in low quantity or of low specific activity when compared to RP4 cRNA.

Figure 3. Quantitative Incorporation of Radioactivity into cRNA by Varying Amounts of Enzyme.

A one ml cRNA reaction mixture was prepared as described by Ketner and Kelly (1976). The reaction mixture was split into four 0.2 ml aliquots and different amounts of enzyme were added to each. At timed intervals a 10 µl sample was removed from each reaction mixture and spotted on a filter. Filters were washed with 5% TCA, rinsed in ethanol and precipitated radioactivity determined.

Legend: (○) no enzyme added; (■) 1 µl (3 units) enzyme; (□) 2 µl (6 units) enzyme; (●) 5 µl (15 units) enzyme added.

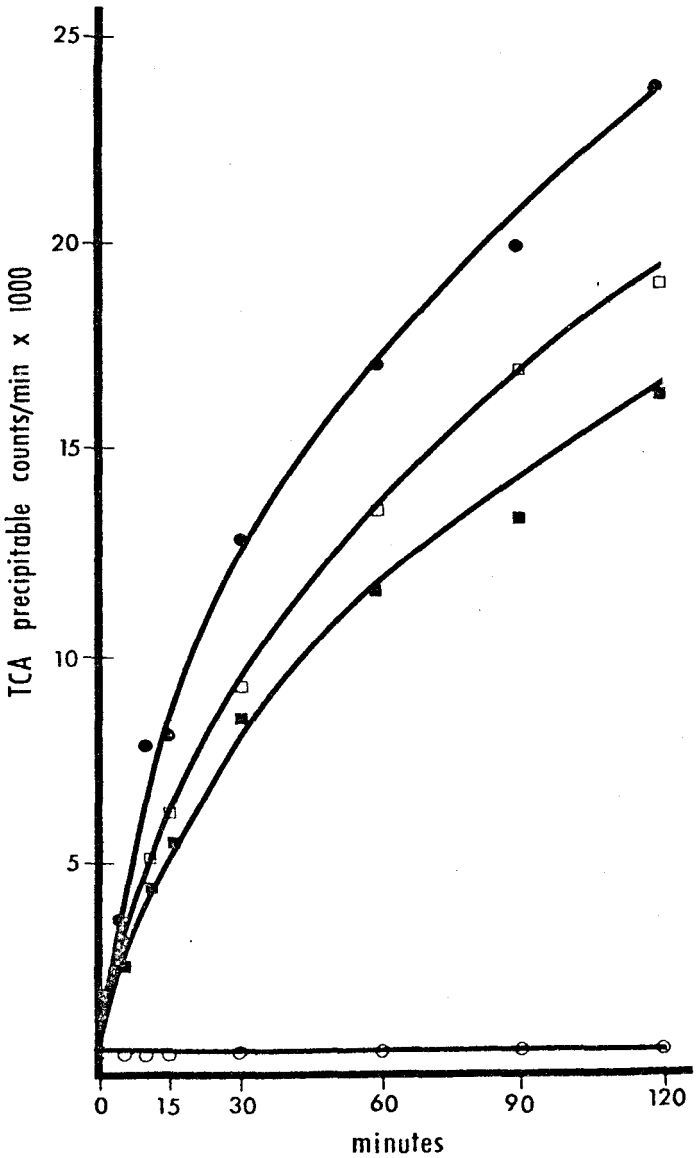
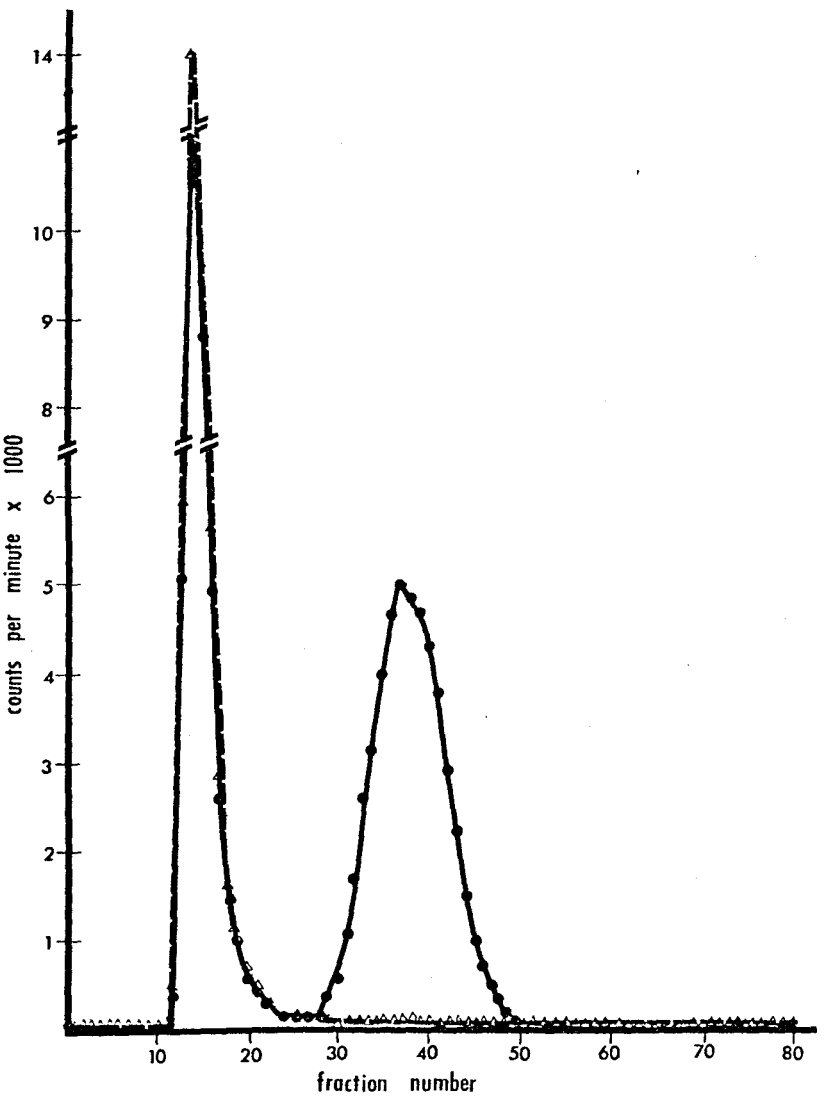

25
20
15
10
5
TCA precipitable counts/min x 1000
0
15
30
60
90
120
minutes

Figure 4. Sephadex G-75 Purification Profile of cRNA Synthesized from an RP4 Template.

A one ml cRNA solution was prepared as described by Ketner and Kelly (1976). Five µl (15 units) of RNA polymerase was added to initiate synthesis. After 5 h the reaction was halted by addition of DNase. The cRNA solution was extracted with phenol and then with diethyl ether. This solution was then layered on a 1.5 x 24 cm Sephadex G-75 column and 15 drop fractions collected from the column. A 10 µl sample from each fraction was spotted on a filter disc, washed with 5% TCA, rinsed with ethanol and TCA precipitable radioactivity determined (Δ). A 10 µl sample of each fraction was spotted on a filter disc and total radioactivity determined (●) without TCA treatment.


14
10
9
8
6
5
4
3
2
1
counts per minute x 1000
10
20
30
40
50
60
70
80
fraction number

The input radioactivity could have been incorporated into an increasingly larger product or into many short strands of cRNA. The size of the cRNA product was analyzed on neutral sucrose gradients. The sucrose profiles revealed that the cRNA product was heterogeneous in size at various time periods rather than exclusively larger in size as incubation time increased (Figure 5).

Colony lysis. Colonies of both A. tumefaciens and E. coli were prepared for filter hybridization with their respective radiolabeled cRNA preparations. The E. coli colonies were easily lysed in situ. However, A. tumefaciens colonies grown on filters were difficult to lyse. Lysis never appeared complete when colony residues were examined by light microscopy. An alternate lysis method for Agrobacterium strains was to suspend a colony of cells in 0.2 ml of the lysis buffer and incubate the suspension at 37°C until the lysate had cleared. Fifty µl of this cell lysate was then spotted on a filter and preparation of the filter for hybridization continued as described from the denaturation of DNA to the end of the procedure.

Hybridization evaluation. To test the efficacy of the technique, RP4 cRNA was hybridized in situ with lysates of E. coli and A. tumefaciens strains harboring and lacking RP4. The extent of cRNA/DNA filter hybridization with the purified RP4 cRNA sample was evaluated by both fluorography (Table 6) and scintillation spectrometry (Table 7). Both methods of detection show the RP4 cRNA to hybridize preferentially with lysates of colonies which harbored RP4. This was evidenced by the greater number of radioactive counts bound to particular lysates. The

Figure 5. Sucrose Profiles of cRNA Product Synthesized from a pAt-B6-806 Template.

A one ml cRNA mixture was prepared as described by Ketner and Kelly (1976). Five µl (15 units) of RNA polymerase was added to initiate the synthesis of cRNA. At timed intervals a 75 µl sample was removed from the reaction mixture. Synthesis was halted by addition of DNase and the sample was layered on a 5-20% neutral sucrose gradient and centrifuged at 125,000 x g for 12 h at 4°C. Centrifuge tubes were punctured from the bottom and 8 drop fractions collected. A 10 µl sample of each fraction was spotted on a filter, washed in 5% TCA, rinsed in ethanol and TCA precipitable radioactivity determined: a) 10 min reaction; b) 20 min reaction; c) 50 min reaction and d) 90 min reaction.

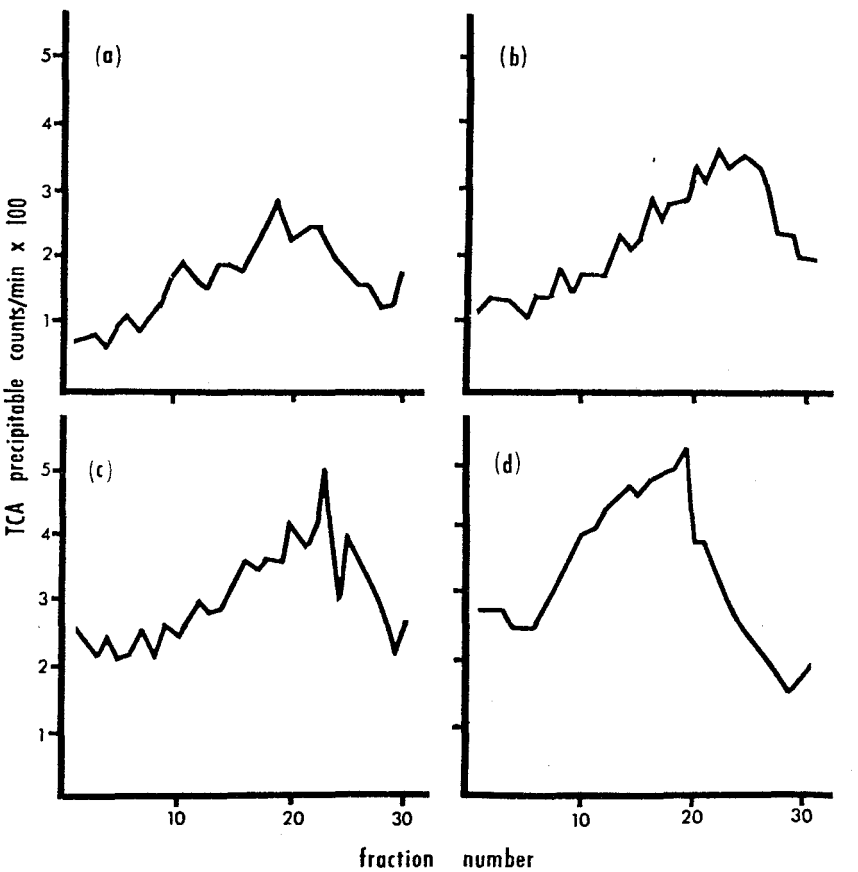

(a)
(b)
(c)
(d)
TCA precipitable counts/min x 100
1
2
3
4
5
10
20
30
fraction number

TABLE 6

Autoradiographic Evaluation of

RP4 cRNA:DNA Filter Hybridization

Filter Bound DNA	Autoradiographic Reaction[a]
C58	-
C58(RP4)	+
NT1	-
NT1(RP4)	+
NT1(pTi-TR1WS)	-
NT1(pTi-TR1WS, RP4)	+
NT1(pTi-B6-Braun)	-
NT1(pTi-B6-Braun, RP4)	+
J53	-
J53(RP4)	+

a - denotes no change in X-ray film, + denotes darkened X-ray film, based on three trials

TABLE 7

Evaluation of RP4 cRNA:DNA Filter Hybridization

by Scintillation Spectrometry

Filter-Bound DNA	Count per Minute[a]
None	51
NT1	24
NT1(RP4)	109
C58	38
C58(RP4)	127
NT1(pTi-TR1WS)	59
NT1(pTi-TR1WS, RP4)	140
NT1(pTi-B6-Braun)	72
NT1(pTi-B6-Braun, RP4)	72
J53	155
J53(RP4)	500

a all sample cpm have been corrected for background

only exception is the low number of counts bound by the lysate of strain NT1(pTi-B6-Braun, RP4). It is possible that this strain did not lyse completely, a condition which would certainly interfere with RP4 cRNA hybridization.

Colony lysates of strains B6-806, B6-Braun, TR1WS and A217 were prepared and filter hybridization with pAt-B6-806 cRNA evaluated. The cRNA used in these experiments did not show consistent preferential hybridization with lysates of colonies which harbored pAt-B6-806 (Table 8). The number of radioactive counts reported hybridized to a filter bound lysate in Table 8 is the average of at least 2 determinations from the same filter.

As reported above, the pAt-B6-806 cRNA product was of low specific activity compared to the RP4 cRNA. All cRNA synthetic reactions using a pAt-B6-806 template were performed with a different lot number RNA polymerase preparation than was used in synthesis of RP4 cRNA. It was possible that the RNA polymerase preparation used in pAt-B6-806 cRNA synthesis was of lower specific activity or inactivated causing a poor copy of pAt-B6-806 cRNA to be synthesized and responsible for the inconsistent or negative cRNA/DNA filter hybridization results (Table 8). In order to test the polymerase preparation, parallel cRNA reaction mixtures were set up, one using pAt-B6-806 as template, the other using RP4. In the first reaction, less than 6% of the input radioactivity was incorporated in pAt-B6-806 cRNA, in the second reaction greater than 30% of the input radioactivity was incorporated in RP4 cRNA. Clearly the activity of the polymerase was not in question.

It was possible that the RNase was removing cRNA that was hybri-

TABLE 8

Evaluation of pAt-B6-806 cRNA:DNA Filter Hybridization by Scintillation Spectrometry

Filter Bound DNA	cpm bound without RNase Treatment	cpm bound after RNase Treatment[a]	
		Exp. #1	Exp.#2[b]
None	139	2	0
B6-806	630	106	15
B6-Braun	687	41	10
TR1WS	252	48	24
A217	231	120	20

a all sample cpm have been corrected for background

b the experiment was repeated on a second day

dized to the filter lysates. A cRNA:DNA filter hybridization was performed and the filter was washed to remove any unbound single stranded cRNA. RNase treatment of the filter to remove cRNA that was nonspecifically bound to the colony lysates was omitted and a greater amount of cRNA remained bound to all of the lysates (Table 8, column 1). After RNase treatment (Table 8, column 3) pAt-B6-806 cRNA hybridization with filter bound positive control colony lysates of B6-806 and A217 was not detected. The pAt-B6-806 cRNA was not expected to hybridize with colony lysates of strains TR1WS and B6-Braun. When RNase treatment was omitted, the number of counts bound with these negative control colony lysates was greater than expected, but after RNase treatment the amount of radioactivity detected was low as expected (Table 8, column 3).

It was concluded that a good copy RNA from pAt-B6-806 could not be made in this fashion. Results indicated the pAt-217 cRNA was unable to hybridize sufficiently with control colony lysates containing complementary sequences. It is probable that the A. tumefaciens plasmid was simply a poor template for the E. coli RNA polymerase. However, it is also possible that the problem was still with the colony lysis. Further application of this technique for detection of NT1(pTi-B6-Braun, pAt-A217) by in situ hybridization was abandoned.

Characterization of the B6 strains. The direct testing of the hypothesis that host specificity markers were located on the plasmids of the B6 strains could not be accomplished. The hypothesis that differences in tumorigenicity of the B6 strains could have been accounted for by differences in the plasmid content and that host specificity

markers might have been located on the plasmids of the strains remained to be tested. It was considered possible that other B6 strains existed that exhibited the same tumorigenicity as strain B6-Braun and strain TR1WS. Indirect evidence in support of the hypothesis would be gained if such strains could be collected and tested for plasmid content. Group B6 A. tumefaciens strains were collected from other laboratories (Table 1) and characterized with respect to a number of different traits.

3-ketolactose test. It has been established that only members of the genus Agrobacterium are able to produce ketoglycosides from the corresponding disaccharides (Bernaerts and De Ley, 1963). As the B6 strain has been frequently subcultured and widely studied it was necessary to be sure all strains surveyed were Agrobacterium. As shown in Table 9, all strains were 3-ketolactose positive.

Bacteriophage studies. In 1955, Beardsley described the phenomenon of lysogeny in A. tumefaciens strain B6. Bacteriophage PS8, sometimes referred to as Omega phage, was identified as the endogenous phage of the B6 strain. Members of the B6 group should either exhibit lysogeny or sensitivity to bacteriophage PS8.

All the strains were tested for lysogeny and for sensitivity to bacteriophage PS8 (Table 9). Twelve of the 20 B6 strains tested were sensitive to authentic PS8 bacteriophage. Of those 8 strains which failed to plaque PS8, 7 were found to be lysogenized. One of the 20 strains tested, B6-Heb, failed to plaque PS8. Further, supernatant from this strain showed no lytic activity when tested against strain

TABLE 9

Characteristics of *Agrobacterium* Strains

Strain	3-keto-lactose	Bacteriophage Study		octopine utilization	Tumorigenicity		% Sequence homology with[a]	
		sensitivity	lysogeny		carrot	Kalanchoe	pAt-B6-806	pTi-B6-806
B6-806	+[b]	±	-	+	+	+	100	100
11156	+	+	-	+	+	+	100	100
11157	+	+	-	+	+	+	100	100
11158	+	+	-	+	+	+	100	100
23308	+	+	-	+	+	+	100	100
B6-Moore	+	+	-	+	+	+	100	100
B6-806(PS8)	±	-	+	+	+	+	nd[c]	nd
B6-Schil	±	-	+	+	+	+	100	100
B6-W	+	-	+	+	+	+	100	100
B6-Tourneur	+	-	+	+	+	+	100	100
B6-Heb	+	-	-	+	+	+	100	100
B6-Braun	+	+	-	+	+	-	0	100
B6	+	+	-	+	+	+	0	100
B6-Matthysse	+	+	-	+	+	+	0	100
B6-S	+	+	-	+	+	+	0	100
B6-6	+	+	-	+	+	+	0	100
B6-M	+	-	+	+	+	+	0	100
TR1WS	+	-	+	+	+	-	0	100
A217	+	+	-	-	-	-	100	0
B6-Man	+	-	+	-	-	-	100	0

a determined by solution hybridization as described in Materials and Methods
b + is positive reaction, -, no reaction, ±, weak reaction
c nd - not determined

B6-806. Strain C58 and its NT1 derivatives are not B6 strains and are, as expected, immune to PS8. The transformants of NT1, NT1(pTi-B6-Braun) and NT1(pTi-TR1WS) were also immune to PS8 indicating the Ti-plasmids did not transfer bacteriophage sensitivity to strain NT1.

Octopine utilization and virulence. Most tumorigenic strains of Agrobacterium demonstrate the ability to utilize either octopine or nopaline (Bomhoff et al., 1976). The ability to utilize these unusual amino acids has been shown to be a characteristic coded for by the Ti-plasmid (Bomhoff et al., 1976). The B6 strains are known octopine utilizers (Montoya et al., 1977; Sciaky et al., 1978) so inability to grow on medium containing octopine as the sole carbon and nitrogen source is suggestive of a B6 strain that has lost the Ti-plasmid. All of the strains tested were found to be octopine utilizers with three exceptions (Table 9, 10). Strain A217 was unable to utilize octopine as sole carbon and nitrogen source. This B6-806 derivative was originally selected by its inability to utilize this opine and does not contain a Ti-plasmid (Sciaky et al., 1978). A second strain, C58, is a known nopaline utilizer (Watson et al., 1975). Lastly, B6-Man was found to be unable to utilize octopine. This strain has a history of virulence (Lippincott, personal communication) and therefore should be able to utilize octopine. It was possible that this strain had lost the Ti-plasmid during storage.

Virulence tests revealed that two B6 strains, B6-Braun and TR1Ws, were virulent on sunflower and carrot, but avirulent on Kalanchoe. The A. tumefaciens B6 group strains were tested for their ability to induce

TABLE 10

Characteristics of Strain NT1 Transformants

Strain	3-keto lactose	Bacteriophage Study sensitivity	lysogeny	octopine utilization	Virulence Study carrot	Kalanchoe	Plasmid Content pAt-B6-806	pTi-B6-806
C58	+[a]	-	-	-	+	+	nd[b]	nd
NT1	+	-	-	-	-	-	-	-
B6-Braun	+	+	-	+	+	-	-	+
TR1WS	+	-	+	+	+	-	-	+
NT1(pTi-B6-Braun)	+	-	-	+	+	+	-	+
NT1(pTi-TR1WS)	+	-	-	+	+	-	-	+

a + is positive test, -, negative test

b nd = not determined

tumors on carrot discs and Kalanchoe leaves to see if any of the strains exhibited a similar limited host range. All B6 strains tested were virulent on both host plants with the following exceptions (Table 9). Strains B6-Braun and TR1WS were virulent on carrot discs, but were unable to produce tumors on Kalanchoe. Strains A217 and B6-Man were unable to induce tumors on either plant. Strain NT1(pTi-TR1WS) possessed the same virulence pattern on carrot discs and Kalanchoe leaves as strain TR1WS (Table 10). This indicated that the pTi-TR1WS virulence genes were expressed in strain NT1. Surprisingly strain NT1 (pTi-B6-Braun) unlike its parent, strain B6-Braun, expressed virulence on Kalanchoe leaves (Table 10). Implications of this are discussed later.

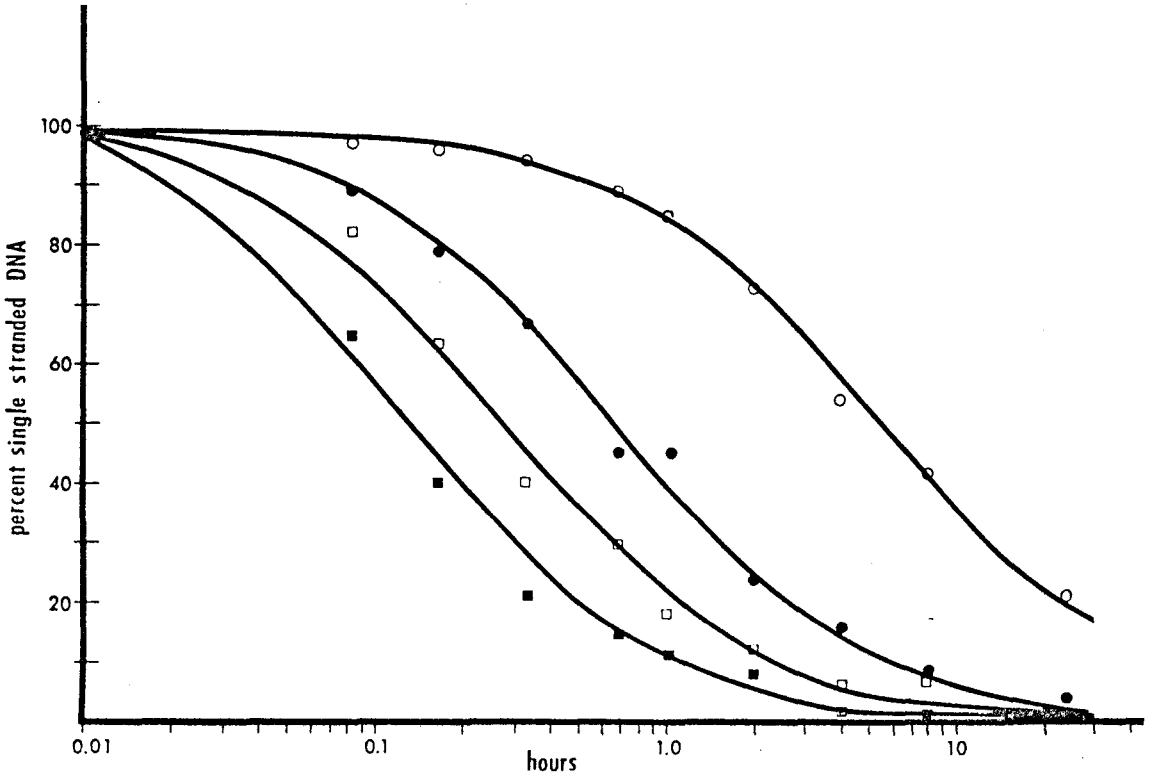
Plasmid analysis. The plasmid content of the B6 strains was determined by DNA:DNA solution hybridization. Tritiated pTi-B6-806 was isolated from strain A277, which contains only the Ti plasmid of strain B6-806 and tritiated pAt-B6-806 was isolated from strain A217, a derivative of strain B6-806 harboring only the cryptic plasmid (Sciaky et al., 1978).

In order to determine the concentration of unlabeled driver DNA to be used in the hybridizations standard curves were first prepared. Such curves (Figure 6) were constructed first by renaturing radiolabeled pAt-B6-806 probe in the presence of unlabeled total A217 driver DNA. The concentrations of driver DNA used were 500 µg/ml, 1 mg/ml and 2 mg/ml. In the presence of A217 driver DNA, a higher concentration of homologous sequences was present. This allowed renaturation of the probe to

Figure 6. Renaturation Kinetics of A. tumefaciens A217 Plasmid DNA in the Presence of Varying Concentrations of Strain A217 Bacterial DNA.

Aliquots of sheared bacterial DNA were evaporated to dryness at 37°C under a stream of air and redissolved in a solution of ^{3}H-probe DNA (17 x 10^{3} cpm/µg DNA; 2.7 µg DNA/ml) in 0.15 M PB. This mixture was drawn up in 10 µl capillary tubes and the tube ends flame sealed. The DNA was heat denatured at 105°C for 7 min and capillaries were then transferred to a 67°C water bath. Renaturation of DNA was allowed to proceed for timed intervals before halting the reaction by chilling the capillaries in iced water. Contents of each capillary was diluted in 1 ml 0.15 M PB and loaded onto hydroxylapatite columns. The single stranded fraction was eluted from the column with 0.15 M PB washes and the double stranded fraction was eluted with 0.3 M PB. The radioactivity in each sample was evaluated by liquid scintillation counting and the single stranded cpm divided by the sum of the single stranded and double stranded cpm to obtain a value for percent single stranded. This value was plotted against time.

Probe DNA was allowed to reassociate with the following concentrations of sheared A217 total DNA samples: (○) probe DNA alone, (●) 500 µg/ml, (□) 1 mg/ml and (■) 2 mg/ml.


100
80
60
40
20
percent single stranded DNA
0.01
0.1
1.0
10
hours

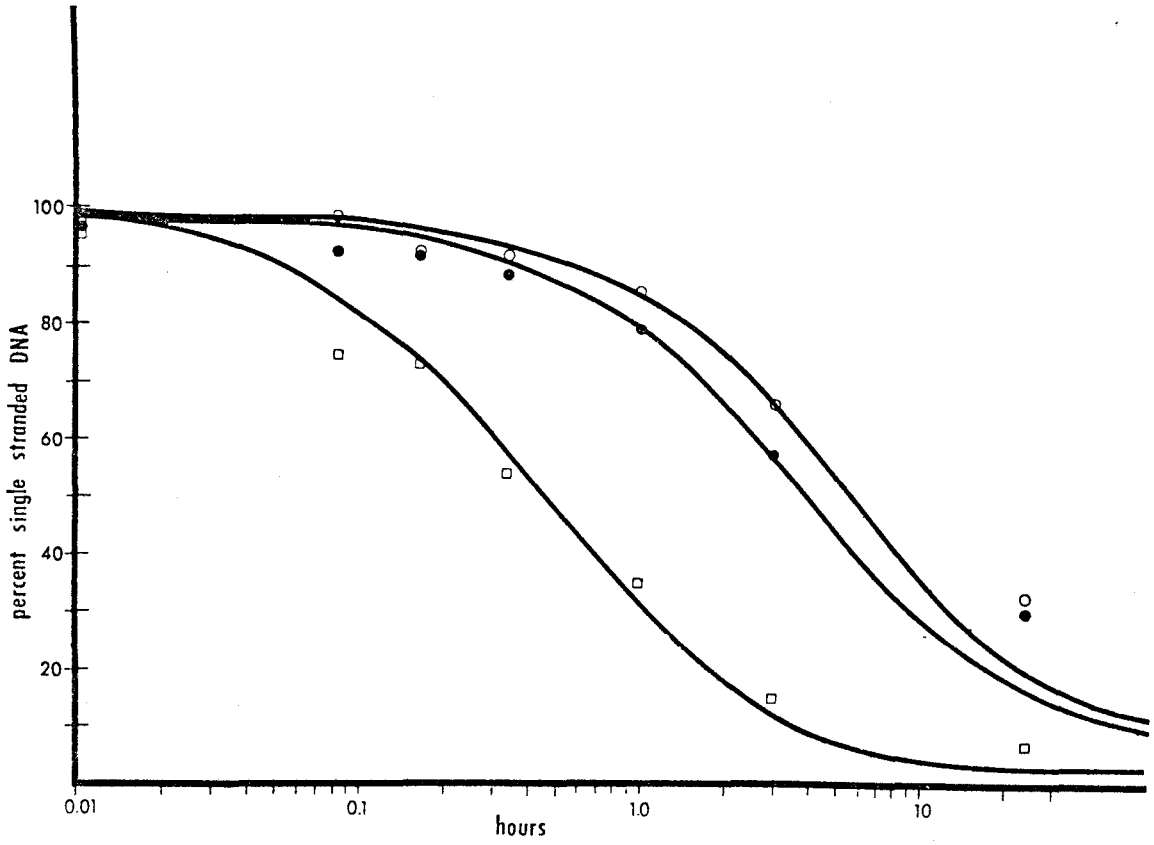
reach completion in a shorter length of time. The t1/2, or time at which 50% of the probe was renatured, was reduced as the concentration of unlabeled total DNA was increased.

It was determined from the standard curves that succeeding hybridizations of labeled pAt-B6-806 or pTi-B6806 probe with B6 strain driver DNA would best be performed at driver DNA concentrations of 1 mg/ml.

Examples of renaturation curves obtained by solution hybridization are shown in Figure 7. Three cases are illustrated: first probe self-renaturation; second, probe renaturation with driver DNA with homologous sequences and third, probe renaturation with driver DNA without homologous sequences. Results of the renaturation experiments are summarized in Table 9. The presence of pTi-B6-806 correlated with the ability of the strains to utilize octopine as the sole carbon and nitrogen source as well as the ability to form tumors on plants. The presence of the pAt-B6-806 plasmid was not necessary for bacterial strains to be virulent and Kalanchoe leaves, as was first suspected. Five of the B6 strains tested did not harbor pAt-B6-806, but were able to produce tumors on Kalanchoe leaves. This indicated that the pAt-B6-806 plasmid was not necessary for the strains to be virulent on Kalanchoe.

Figure 7. Renaturation Kinetics of pAt-B6-806 Plasmid DNA in the Presence of Bacterial DNA Lacking pAt-B6-806 Sequences.

Experiments were performed as described in Figure 6. Tritium labeled plasmid DNA was allowed to reassociate in the presence of: (○) 1 mg/ml J53 DNA, (●) 1 mg/ml B6-Braun DNA and (□) 1 mg/ml B6-806 DNA.


100
80
60
40
20
percent single stranded DNA
0.01
0.1
1.0
10
hours

DISCUSSION

Sciaky et al. (1978) demonstrated the presence of two plasmids in strain B6-806 through comparison of restriction endonuclease fragment patterns. These plasmids are a Ti-plasmid, designated pTi-B6-806, and a cryptic plasmid which carries no known markers, designated pAt-B6-806 (Sciaky, 1977). These plasmids have been segregated into strains A277, which carries only the pTi-B6-806 (Chilton et al., 1977) and A217, which harbors pAt-B6-806 (Sciaky et al., 1978). Our analysis of plasmid content by solution hybridization (Table 9) confirms the work of Sciaky et al. (1978) demonstrating that strain B6-806 contains plasmid sequences homologous to both pTi-B6-806, isolated from strain A277, and pAt-B6-806, isolated from strain A217.

The two B6-806 plasmid types could not be distinguished on the basis of molecular mass calculated from contour length measurements of open circular molecules. The electron microscopy study reveals the plasmids of strain B6-806 have an average molecular mass of 126×10^6 dal (Table 2), but two distinct size classes were not observed (Figure 2). Currier and Nester (1976a) were also unable to detect two separate size classes for the plasmids and reported a mass of $125 \pm 8 \times 10^6$ dal for the strain B6-806 plasmids. Strain TR1WS contains a plasmid whose molecular mass of 121×10^6 dal is approximately the same as the average mass of the strain B6-806 plasmids (Table 2). Farrand (personal communication) observed 100% homology when plasmid DNA from strain TR1WS was hybridized with an excess of strain B6-806 plasmid preparation, but

only 75% homology when a strain B6-806 plasmid preparation was hybridized with excess plasmid DNA from TR1WS. Some plasmid sequences present in strain B6-806 are absent from strain TR1WS and a possible explanation is that a deletion of up to 25% of the strain B6-806 plasmid complement has taken place in the strain TR1WS plasmid (Farrand, pr. communication). This theory is discounted by the fact that the molecular mass of the plasmids was comparable. Solution hybridization revealed that strain TR1WS harbors the Ti-plasmid, but contains no sequences homologous to pAt-B6-806. The difference in sequence homology observed by Farrand is most probably due to the absence of the cryptic plasmid.

The Ti-plasmid from strain B6-Braun has the same average molecular mass, 126 x 10^6 dal (Table 2), as the plasmids from strain B6-806. It had not been previously established if strain B6-Braun contained 1 or 2 plasmids. The solution hybridizations demonstrate that strain B6-Braun lacks sequences homologous to pAt-B6-806 (Table 9). It does not appear that a deletion in the Ti-plasmid DNA has taken place. Strains B6-Braun and TR1WS are virulent on all plant hosts tested with the exception of Kalanchoe.

Farrand (pr. communication) has suggested that host specificity markers are carried on the Agrobacterium plasmids and hypothesized that the host specificity markers for Kalanchoe virulence are carried on a deleted segment of the Ti-plasmid in strains TR1WS and B6-Braun. Since no evidence for deletion was found it may be possible that the host specificity markers hypothesized are located on pAt-B6-806.

The plasmid contour length measurements obtained for pAt-B6-806 isolated from strain A217 are smaller than those for pTi-B6-806 (Table

2) although pAt-B6-806 from strain A217 should be identical to pAt-B6-806 from strain B6-806 (Sciaky et al., 1978). Data in Table 2 agrees with the literature value of Stanish and Ortiz (1976) for the molecular mass of the R-factor RP4. This demonstrates that the method of molecular mass determination utilized is reliable and implies that values obtained for all molecules measured were accurate. It is possible that the A217 plasmid preparation did not spread sufficiently to yield well laid out, relaxed open circular molecules and pAt-B6-806 appeared smaller than it actually is.

Strain B6-806 is virulent on Kalanchoe and contains two types of plasmids (Sciaky et al., 1978; Table 10) while strains B6-Braun and TR1WS are avirulent on this plant and lack pAt-B6-806 sequences. One way to test the hypothesis that Kalanchoe host specificity markers are located on the pAt-B6-806 plasmid is to transfer pAt-B6-806 into strain B6-Braun and strain TR1WS. Such strains should gain virulence on Kalanchoe. Unfortunately, these two strains proved to be poorly transformable (Table 3).

These transformation experiments were performed the day after Form I RP4 was purified so it is unlikely that low level of transformation was due to poor quality or low quantity of DNA. It was suspected that transformation of strain B6-Braun with pAt-B6-806 would occur at very low frequency and it was consequently doubtful that the strain B6-Braun(pAt-B6-806) transformants could be isolated.

Not all A. tumefaciens strains are as poorly transformable as strains B6-Braun and TR1WS appear to be. It is possible to avoid these problems by performing sequential transformations of the transformable

strain NT1. The first step would be to transform NT1 with pTi-B6-Braun or pTi-TR1WS. These transformants should be avirulent on Kalanchoe. These strains could then be transformed with the cryptic plasmid, pAt-B6-806. If the hypothesis is correct, the transformants containing both a Ti-plasmid and the cryptic plasmid should exhibit virulence on Kalanchoe.

The isolation of NT1(pTi-B6-Braun) and NT1(pTi-TR1WS) was accomplished (Table 3) and these strains were to be transformed with pAt-B6-806. Plasmid pAt-B6-806 carries no selectable marker so individual screening of all recipient cells is necessary for identification of the desired transformant. As transformation takes place at very low frequency, less than 1×10^{-8}, this would be an impractical task. Kretschmer et al. (1975) describe a cotransformation which utilizes a selectable plasmid as an indicator to identify cells that have acquired a second plasmid that is otherwise nonselectable. With this procedure, if the molar ratio of selectable to nonselectable plasmids is 10^{-3} or less, greater than half of the selected transformants are found to have acquired the unselected plasmid as well. Cotransformation of NT1(pTi-B6-Braun) with pAt-B6-806 and RP4 was performed to increase the likelihood of detecting a pAt-B6-806 recipient.

The systems used for synthesis of complementary RNA and for detection of specific DNA sequences by filter hybridization were defined using RP4 as a template for cRNA synthesis and an RP4 cRNA probe for filter hybridization (Tables 7, 8). When cRNA synthesis experiments were repeated with pAt-B6-806 as a template the percent incorporation of radiolabel in the pAt-B6-806 cRNA product was not as high as that of

RP4 cRNA (data not shown). The A. tumefaciens DNA may not be an accessible template for E. coli RNA polymerase due to the conformation of the DNA. It is also possible that the RNA polymerase uncovered a stop codon soon after binding to the pAt-B6-806 DNA so synthesis of macromolecules was not accomplished. The pAt-B6-806 cRNA was used in in situ hybridization in spite of low specific activity.

The pAt-B6-806 cRNA was hybridized with prepared control filters. Some of the colony lysates fixed to these filters contained plasmid DNA sequences that were homologous to pAt-B6-806 and other colony lysates did not contain plasmid sequences homologous to pAt-B6-806. Data shown in Table 8 indicates the pAt-B6-806 cRNA did not consistently demonstrate preferential binding to colony lysates that were known to contain pAt-B6-806 sequences. When experiment #1 (Table 8) was performed the pAt-B6-806 cRNA hybridized preferentially with filter bound lysates known to contain sequences homologous to pAt-B6-806 and did not bind to strain B6-Braun or strain TR1WS filter bound lysates. These results were expected. However, when the experiment was repeated on the following day (Experiment #2, Table 8), the pAt-B6-806 cRNA did not bind to any of the colony lysates. It is possible that lysis of the cells was incomplete and DNA was inaccessible for hybridization with the pAt-B6-806 cRNA. Radiolabel was not incorporated into cRNA product very efficiently as discussed earlier. Perhaps the amount of radioactivity applied to the filter was too low to be detected. A pAt-B6-806 cRNA:filter bound DNA hybridization was performed as described and the total amount of tritiated cRNA applied to the filter was determined. The filter was not

treated with RNase to remove excess cRNA prior to determination of radioactivity on the filter. The data in Table 8 reveals that an easily detectable number of counts was applied to the filter. Low specific activity of the cRNA alone was not responsible for the negative results of experiment #2 presented in Table 8. Perhaps the cRNA misspaired with the filter bound DNA and the hybridized cRNA was cleaved when RNase was applied to remove unbound cRNA. The attempts to identify pAt-B6-806 sequences by in situ hybridization were unsuccessful making it virtually impossible to screen the NT1 transformants for the cryptic plasmid.

Since transformants containing both pTi-B6-Braun and pAt-B6-806 could not be identified by in situ hybridization the location of host specificity markers on the plasmids could not be directly proven. There was, however, a way to test the hypothesis which avoided the problems associated with constructing and identifying a strain that harbored the two plasmids. Since strain B6-806 contains both a Ti-plasmid and a cryptic plasmid it is probable that other B6 strains harbor these two plasmids. If it could be shown that absence of a cryptic plasmid yields a strain with a narrowed host range, then it is possible that the virulent strains of B6-Braun and TR1WS are avirulent on Kalanchoe due to loss of the cryptic plasmid. A comparative study of B6 strains was undertaken in order to characterize the strains and to attempt to correlate plasmid content with tumorigenicity.

The virulent B6 prototype strain has been subcultured and widely studied. When bacterial cultures were collected for this survey it was necessary to be sure all were A. tumefaciens B6 strains. Parameters

surveyed included 3-ketolactose production, octopine utilization, bacteriophage PS8 sensitivity, lysogeny by PS8, virulence on carrot and Kalanchoe and plasmid content.

The production of 3-ketolactose is a characteristic of Agrobacterium strains described by Bernaerts and De Ley in 1963. Only members of the genus Agrobacterium produce 3-ketoglycosides from the corresponding disaccharides. All of the strains studied were shown to be 3-ketolactose positive Agrobacterium strains (Table 9, 10).

Although strain B6 was described as lysogenized in 1955, only 7 of the 20 strains surveyed were lysogenized (Table 9). This indicated that it was not unusual for the bacteriophage to be lost. One strain, B6-Heb, was neither lysogenized nor sensitive to PS8. It is possible that this strain is a nonlysogenic, PS8 resistant mutant of strain B6. An alternative is that strain B6-Heb carries a defective prophage which cannot replicate, but which can prevent superinfection with bacteriophage PS8. In all other characteristics examined, strain B6-Heb resembles strain B6-806 (Table 9). Evidence that strain B6-Heb is a B6 strain includes the fact that strain B6-Heb harbors plasmids 100% homologous with pTi-B6-806 and pAt-B6-806.

Not all of the B6 strains harbor pAt-B6-806 (Table 9) despite their similar background; for example, strains 11156, 11157 and 11158 differ from parental strain B6-Braun in plasmid content. Strain B6-Braun lacks pAt-B6-806 while the ATCC strains contain this cryptic plasmid. It is possible for any bacterial strain to change by loss of genetic information, plasmid and phage, during passage and maintenance in different laboratories, therefore, it is possible that pAt-B6-806

was lost from seven of the B6 strains after they were acquired by various laboratories. However, it is unlikely that the subcultured strains would gain genetic information, i.e. the cryptic plasmid, during passage. It would be interesting to know the plasmid content of the B6 parent of strain B6-806, at the time that it was first subcultured in various laboratories.

When correlating plasmid content with pathogenicity (Table 10) it is evident that the Ti-plasmid is essential for virulence. Strains which lack the Ti-plasmid are avirulent (Table 9). This observation confirms that of numerous other workers (Van Larebeke et al., 1974, 1975; Watson et al., 1975; Zaenen et al., 1974; Chilton et al., 1977, 1978; De Picker et al., 1978). Many of the B6 strains harbor pAt-B6-806, but genetic information coded for by pAt-B6-806 is not essential for bacterial pathogenicity. Seven virulent B6 strains lacked pAt-B6-806 sequences (Table 9) although the hybridization experiments would have detected pAt-B6-806 sequences whether plasmid sequences existed extrachromosomally or were integrated in the host chromosome. Two of these strains were avirulent on Kalanchoe while the other 5 were virulent on Kalanchoe (Table 9). The cryptic plasmid does not appear to play a role in virulence so its function is still unknown.

It was suspected that host specificity markers were located on the cryptic plasmid. Since this is shown not to be the case there must be some other explanation for the inability of strain B6-Braun and strain TR1WS to induce tumor formation on Kalanchoe.

Tumorigenicity transferred when the Ti-plasmid was transferred to strain NT1 (Table 10). This confirms the observation of other workers

(Van Larebeke et al., 1975). The pTi-TR1WS and pTi-B6-Braun virulence genes are expressed in strain NT1 as evidenced by the ability of transformants, NT1(pTi-B6-Braun) and NT1(pTi-TR1WS), to form tumors on carrots (Table 10). Surprisingly, strain NT1(pTi-B6-Braun) was able to induce tumor formation on Kalanchoe leaves (Table 10) although both strain NT1 and strain B6-Braun are nontumorigenic on this host plant.

It is not known exactly what has enabled strain NT1(pTi-B6-Braun) to form tumors on Kalanchoe. While evidence collected indicates the Ti-plasmid to be necessary for tumor induction, there is no indication that plasmid genes are sufficient (Currier and Nester, 1976). It has also been suggested that mutations on the chromosome are capable of interfering with pathogenicity (Sciaky et al., 1978). Sciaky et al. (1978) have also reported that transfer of a plasmid from an avirulent mutant, 5GlyFeAvir derived from the virulent A6 strain, to another recipient strain resulted in a virulent transconjugant. Apparently the 5GlyFeAvir plasmid was fully competent and a chromosomal mutation rendered 5GlyFeAvir avirulent (Sciaky et al., 1978). Plasmid and chromosomal genes must be necessary for bacterial pathogenicity. Strain B6-Braun exhibits a limited pathogenicity while an NT1 strain which harbors pTi-B6-Braun has a broader host range, including Kalanchoe. Identification of the difference between strain NT1(pTi-B6-Braun) and strain B6-Braun could provide insight into the tumor induction process. On the other hand, strain TR1WS and its transformant, NT1(pTi-TR1WS), are both tumorigenic on carrot discs but unable to induce tumors on Kalanchoe. Both parental strains B6-Braun and TR1WS have a limited host range, but strain B6-Braun appears to contain a normal Ti-plasmid. Comparative study of

strain B6-Braun and strain TR1WS would be valuable since these strains appear to be avirulent on Kalanchoe for different reasons. Restriction endonuclease analysis of the Ti-plasmids from strain B6-806, B6-Braun, TR1WS and the transformants NT1(pTi-B6-Braun) and NT1(pTi-TR1WS) may provide useful information.

On the basis of the information presented above, several conclusions can be drawn. First, the pTi-TR1WS and pTi-B6-Braun virulence genes are expressed in NT1 as exemplified by the observation that derivatives of NT1 harboring these elements gain the ability to form tumors on carrots. Second, all virulent strains harbored pTi-B6-806 and many strains, 12 out of 19 surveyed, also carry pAt-B6-806. Third, the presence of the cryptic plasmid is not essential for any known virulence functions since strains that do not harbor the pAt-B6-806 plasmid demonstrate virulence. In addition, strain A277, which contains only pTi-B6-806, has been observed to be able to induce tumor formation on Kalanchoe.

The importance of the Ti-plasmid for pathogenicity has been repeatedly demonstrated (Zaenen et al., 1974; Watson et al., 1975; Currier and Nester, 1976; Chilton et al., 1977). Many strains of Agrobacterium harbor more than one plasmid, but only the Ti-plasmids appear to be essential for tumor-inducing ability. The origin and role of cryptic plasmids, plasmids with no known markers, in agrobacteria has not yet been defined. The cryptic plasmid appears to be nonessential and may never have had any role in tumorigenicity. If these cryptic plasmids do not code for some advantageous characteristics, they will not be maintained in the strains. The fact that 7 out of 19 B6 strains

surveyed do not harbor this cryptic plasmid may indicate that it is being eliminated from the B6 strains.

SUMMARY

Although pathogenic A. tumefaciens strains often harbor more than one plasmid (Currier and Nester, 1976a; Sciaky et al., 1978), all plasmids harbored by a strain are not necessarily Ti-plasmids (Currier and Nester, 1976a). Some Agrobacterium plasmids do not code for any known functions. The origin of these cryptic plasmids is unknown and little study has been devoted to determining the role these plasmids play in host strains.

There is a difference in the host range of several virulent B6 strains of A. tumefaciens. This difference in pathogenicity might be accounted for by variation in plasmid content. Attempts to construct and test the tumorigenicity of a strain with known composite plasmid content ended when such a new strain could not be identified by in situ hybridization.

In order to examine the role of the B6-806 strain plasmids, twenty existing B6 strains were characterized on the basis of bacteriophage sensitivity, octopine utilization, virulence and plasmid content. While the B6 strain characteristically harbors Omega phage (Beardsley, 1955), only 7 of the 20 B6 strains surveyed were found to be lysogenized with PS8. This indicated that it was not unusual for the bacteriophage to be lost. One strain, B6-Heb, was neither lysogenized nor sensitive to PS8. The ability to utilize octopine or nopaline is a characteristic coded for by the Ti-plasmid (Bomhoff et al., 1976). The B6 strains are known octopine utilizers (Montoya et al., 1977; Sciaky et al., 1978) so

inability to utilize octopine as sole carbon and nitrogen source is suggestive of a B6 strain that has lost the Ti-plasmid. Two of the B6 strains tested were unable to utilize octopine. One strain, A217, was an avirulent derivative of strain B6-806 that had been selected by its inability to utilize octopine. The second strain, B6-Man, was later shown to have lost the Ti-plasmid.

Virulence tests revealed that the strains B6-Braun and TR1WS were virulent on carrot, but avirulent on Kalanchoe. The A. tumefaciens B6 strains were tested for their ability to induce tumors on carrot and Kalanchoe to determine if any of the other strains exhibited a similar limited host range. No such strains were found. The plasmid content of the strains was analyzed and seven of the B6 strains, including TR1WS and B6-Braun, were shown to harbor only pTi-B6-806. Five such strains lacking pAt-B6-806 sequences remained virulent on Kalanchoe. Evidently genetic information coded for by the cryptic plasmid is not essential for pathogenicity on Kalanchoe. There must be some other explanation for the inability of strains B6-Braun and TR1WS to induce tumor formation on Kalanchoe.

The Ti-plasmids from strain B6-Braun and TR1WS, pTi-B6-Braun and pTi-TR1WS, were isolated and examined in an NT1 background. The NT1-(pTi-TR1WS) transformant was tumorigenic on carrot and avirulent on Kalanchoe, as was the TR1WS strain. However, the NT1(pTi-B6-Braun) transformant, unlike strain B6-Braun, was pathogenic on both test plants.

Several statements can be made on the basis of the information gathered. First, the difference in sequence homology of plasmid pre-

parations from strain B6-806 and TR1WS observed by Farrand was not due to a large deletion in TR1WS plasmid DNA, but probably due to the absence of the cryptic plasmid in TR1WS. Second, the pTi-TR1WS and pTi-B6-Braun virulence genes are expressed in NT1 as exemplified by the observation that derivatives of NT1 harboring these elements gain the ability to form tumors on carrots. Third, all virulent strains harbored pTi-B6-806 and many strains, 12 out of 19 surveyed, also carried pAt-B6-806. Fourth, the presence of the cryptic plasmid is not essential for any known virulence functions since strains that do not harbor pAt-B6-806 demonstrate virulence. In addition, strain A277, which contains only pTi-B6-806, has been observed to induce tumor formation on Kalanchoe.

Identification of the difference between strains B6-Braun and NT1(pTi-B6-Braun) would yield insight on the tumor induction process as it appears that both plasmid and chromosomal genes must be necessary for bacterial pathogenicity (Sciaky et al., 1978). Comparative study of strain B6-Braun and strain TR1WS would also be valuable. Since strain B6-Braun appears to harbor a normal Ti-plasmid, these strains appear to be avirulent on Kalanchoe for different reasons.

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APPROVAL SHEET

The thesis submitted by Suzanne E. Hamada has been read and approved by the following Committee:

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The final copies have been examined by the director of the thesis, and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology.

11 April 79
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