JOURNAL OF BACTERIOLOGY, Apr. 1976, p. 157-165

Vol. 126, No. 1
Printed in U.S.A.

Evidence for Diverse Types of Large Plasmids in Tumor-Inducing Strains of *Agrobacterium*

T. C. CURRIER[1] AND E. W. NESTER*

Department of Microbiology and Immunology, University of Washington, Seattle, Washington 98195

Received for publication 30 December 1975

Homology between the large plasmids of 15 pathogenic *Agrobacterium* strains isolated from various parts of the world has been measured and was found to vary over a wide range, from 3 to 100%. Two genetically distinct groups of plasmids can be identified: one closely related to the plasmid of *A. tumefaciens* A6, an octopine-utilizing strain, and the other closely related to the plasmid of *A. tumefaciens* C-58, a nopaline-utilizing strain. The plasmids of four *Agrobacterium* strains do not belong to either group. One of these four strains utilizes octopine, one utilizes nopaline, and two utilize neither. Three strains contained two large plasmids. In one of these strains, the two plasmids were not homologous to one another. Chromosomal homologies for the *Agrobacterium* strains surveyed also vary over a wide range, but do not correlate with plasmid homologies. Neither do plasmid homologies correlate with any numerical classification scheme. The significance of these plasmid homology studies for crown gall tumorigenesis is considered.

Crown gall, a neoplastic disease of dicotyledonous plants, is induced when a pathogenic strain of *Agrobacterium* is introduced into a freshly wounded site in a susceptible plant. Cell transformation requires only a brief period of contact between bacteria and the plant cell (3). The mechanism by which the bacterium mediates this cell transformation is not known.

Circumstantial evidence has implicated a large plasmid in the transformation process. A survey of 11 pathogenic and 8 nonpathogenic strains of *Agrobacterium* showed that a large plasmid is present only in the pathogenic strains (28). Direct evidence that such a large plasmid plays an indispensable role in pathogenicity has more recently been obtained. Loss of pathogenicity in *A. tumefaciens* C-58 was found to occur concomitantly with loss of a large closed circular plasmid (25, 27). Deoxyribonucleic acid (DNA) hybridization studies subsequently proved that plasmid is lost by elimination rather than integration into the bacterial chromosome (27). Further, acquisition of pathogenicity has been found to be due to transfer of a plasmid from a pathogenic donor strain to a nonpathogenic recipient (26, 27). From these data we conclude that in these strains the presence of a large plasmid is required for pathogenicity. Because all pathogenic strains contain a large plasmid we have made the assumption that a large plasmid is required for tumor induction in all *Agrobacterium* strains.

If all *Agrobacterium* strains induce tumors by a common mechanism, then some genes might be common to all large *Agrobacterium* plasmids. In the present study we have used the technique of DNA reassociation in solution to measure the degree of DNA homology between large *Agrobacterium* plasmids as well as the homology between the chromosomal DNA from 14 strains of *Agrobacterium*. The data reveal that the homology between different plasmids varies over a wide range, and that plasmid homology is independent of chromosomal homology.

MATERIALS AND METHODS

Culture media. Stock cultures were maintained at 4 C on 1.5% agar slants containing 0.8% nutrient broth (Difco). Cultures were routinely streaked from the stock cultures onto nutrient agar plates, and single colonies were used to inoculate liquid media.

The synthetic medium most commonly used was AB medium (27), which was sometimes supplemented with 0.05% yeast extract. Biotype 2 organisms (10) were routinely grown in mannitol-glutamate medium (19).

Bacterial strains. The bacterial strains used in this study are described in Table 1. These strains represent three different species of *Agrobacterium*. *A. tumefaciens*, the causative agent of crown gall, induces proliferations of either unorganized or teratomatous tumors on a wide variety of plants. Strains of *A. rhizogenes* are the etiological agents of hairy root disease, evidenced by proliferations of roots which are induced on some plant genera. *A. rubi*

[1] Present address: Department of Crop and Soil Sciences, Michigan State University, East Lansing, Mich. 48824.

TABLE 1. *Source and description of strains*

Strains	Source[a]	Description	Reference
Pathogenic			
Agrobacterium tumefaciens			
27	Kerr	Peach gall, Loveday, S. Australia	19
223	NCPBB	Isolated in New Zealand	12
0362	Haywood	John Innes potting soil, Australia	13
IIBV7	Lippincott	Derived from Stapp's *Chrysanthemum frutescens* strain by Armin Braun	Braun, private communication
C-58	Hamilton	Virulent strain that gives rise to plasmidless nonpathogenic strains when grown at 37 C	9
181	Lippincott	Isolated from a poplar tree tumor by Nellie Brown, 1928	Braun, private communication
A6	Schilperoort	No information available	
B6-806	Lippincott	Phage-sensitive isolate from ultraviolet-irradiated B6	1
CGIC	Moore	Isolated from a pear tumor	23
15955	ATCC	TT134 strain of M. P. Starr	
B2A	Pootjes	Subclone of strain B2 (Stonier)	13
A197	Our laboratory	Plasmid recipient of mating between *A. tumefaciens* 27 and a rifampin- and nalidixic acid-resistant derivative of *A. tumefaciens* NT1	27
AT-1	Lippincott	Dahlia Rabitz 1a, unable to utilize either octopine or nopaline	15
AT-4	Lippincott	Dahlia Rabitz 1b, unable to utilize either octopine or nopaline	15
A. rhizogenes			
TR7	Lippincott (ICPB)	No information available	
A. rubi			
13335	Lippincott (ATCC)	ICPB, TR3; probably E.M. Hildebrand's "boysenberry no. 1" Ithaca, N.Y.	M. P. Starr, private communication
Nonpathogenic			
NT1	Our laboratory	Nonpathogenic derivative of *A. tumefaciens* C-58	27

[a] ICPB, International Collection of Pathogenic Bacteria; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England; ATTC, American Type Culture Collection.

strains are so called because they induce tumors on plants of the genus *Rubus*. In nature, these latter strains induce aerial rather than crown type galls.

Preparation of bacterial lysates. A modification of the neutral lysis technique of Zaenen et al. (28) was used. Sodium dodecyl sulfate was substituted for Sarkosyl NL97 in the procedure and the lysozyme treatment was omitted.

Preparative isolation of plasmid DNA. The details of this procedure will be reported elsewhere (T. C. Currier and E. W. Nester, submitted for publication). Briefly, the procedure consists of shearing and alkaline denaturation of the lysate followed by neutralization and treatment with phenol. The covalently closed circular plasmid was isolated by centrifuging the DNA to equilibrium in cesium chloride density gradients which contained 500 μg of ethidium bromide per ml. The covalently closed circular plasmid which bands at a greater density than noncovalently closed circular DNA was removed. The ethidium bromide was removed by extraction with isopropyl alcohol (27). The plasmid in 0.05 M tris(hydroxymethyl)aminomethane, 0.05 M NaCl, 0.005 M ethylenediaminetetraacetic acid, pH 8.0, was treated with ribonuclease A and subjected to gel filtration (Bio-Gel 1.5, 1 by 23-cm column). Before the ribonuclease treatment, a sample of the plasmid was prepared for electron microscopy by the Kleinschmidt technique (14), and molecular weights were determined by measuring the contour lengths of relaxed plasmid molecules (27).

Isolation of bacterial DNA. Bacterial DNA was isolated from bacterial lysates by a modification of the Marmur procedure (16).

Preparation of radioactive DNA of high specific activity. Unlabeled bacterial DNA and labeled plas-

mid of low specific activity were labeled in vitro to a high specific activity (>5 × 10^6 count/min per μg) by nick translation with DNA polymerase I (20). For labeling of the plasmid to a low specific activity, bacterial cultures were inoculated at 5 × 10^7 cells per ml in mannitol-glutamate or AB medium containing 1 μCi of [^{3}H]thymidine per ml and harvested at 5 × 10^8 cells per ml. Bacterial and plasmid DNA were isolated as described above.

DNA hybridization experiments. All unlabeled DNA samples were sheared with a French pressure cell at 12,000 lb/in^2 to give a double-stranded molecular weight of approximately 1 × 10^6. Radioactively labeled DNA samples were treated with deoxyribonuclease I in the process of in vitro labeling to give a single-stranded molecular weight of 2.5 × 10^5. Unlabeled plasmid from one bacterial strain in distilled H_2O was dried in a jet of air and dissolved in a solution of one of the labeled plasmids in 0.15 M phosphate buffer (0.075 M NaH_2PO_4, 0.075 Na_2HPO_4). The plasmid mixture was heat denatured (102 C, 5 min) and allowed to reassociate for 12 and 24 h at 70 C. The samples were diluted into 1 ml of 0.15 M phosphate buffer and the extent of duplex formation was assayed on hydroxylapatite as previously described (4). The labeled "probe" DNA was at such a low concentration (<0.01 μg/ml) that self-renaturation was only 3.5%. The concentration of the unlabeled plasmid was between 50 and 100 μg per ml, sufficiently high to ensure that the unlabeled plasmid is greater than 99% renatured in 12 h. To show that the extent of duplex formation between the labeled and unlabeled plasmids was complete at 12 h, the percentage of duplex formation was also measured at 24 h. The percentage of duplex formation at 12 and 24 h was within the variability of the assay (±5%). The average of these values was used in subsequent calculations (see below).

Calculation of percentage of DNA homology. The data in Table 2 are given in percentage of DNA homology instead of percentage of duplex formation because the labeled probe DNA did not show 100% duplex formation when renatured with the unlabeled homologous plasmid (values ranged from 64 to 80%). The percentage of homology is calculated by dividing the average percentage of duplex formation for the heterologous hybridization by the average percentage of duplex formation for the homologous hybridization reaction. This calculation defines the percentages of homology for the homologous hybridization reactions as 100% and normalizes the percentage of duplex formation for the heterologous hybridization reactions to this value. The homology data have not been corrected for the self-renaturation of the labeled plasmid, which was approximately 5% in all controls. Such a correction cannot be applied to all reactions uniformly because heteroduplex formation competes with self-renaturation. Thus the greater the homology between two plasmids, the less self-renaturation the labeled plasmid will undergo.

The inability of some of the labeled probe DNA to form duplexes with the unlabeled homologous plasmid is probably due to an initial twofold difference in their molecular weights (the single-stranded molecular weight of labeled probe is half that of the unlabled plasmid). Further breakdown of the high-specific-activity probe DNA as a result of radioactive decay would increase the molecular weight difference between the labeled and the unlabeled DNA strands even more. In support of this explanation we observed that the percentage of duplex formation for the homologous hybridization reaction gradually decreased over the 5-month period in which these experiments were performed. Evidence that the single-stranded molecular weight of the radioactive probe DNA was decreasing with time was obtained by sedimentation analysis.

RESULTS

Molecular weight determinations of *Agrobacterium* plasmids. The molecular weights of the large plasmid from the bacterial strains that were used in the study range from 98 × 10^6 to 158 × 10^6 (Table 2). This range of molecular weights is in general agreement with previously reported values (28). In the course of these studies, it became apparent that many strains contained more than one size class of plasmids. Three strains of *Agrobacterium* contain two large plasmids. *A. tumefaciens* 27 contains plasmids of 127 × 10^6 daltons (127 megadaltons) and 153 megadaltons; *A. tumefaciens* 181 contains plasmids of 114 and 158 megadaltons; and *A. rhizogenes* TR7 contains plasmids of 98 and 140 megadaltons. Many other pathogenic strains of *Agrobacterium* harbor smaller plasmids in addition to a pathogenicity-determining plasmid. For example, *A. tumefaciens* 0362 and CGIC contain plasmids of 30 and 47 megadaltons, respectively. At present there is no evidence that these smaller plasmids play any role in the pathogenicity of these strains. These plasmids occur two to four times more frequently than the larger plasmids in preparations examined by electron microscopy. Whether this represents their relative frequency inside the cell or is merely a bias resulting from the plasmid isolation procedure is not clear. Small plasmid molecules were occasionally seen in plasmid preparations from other strains but at such a low frequency that less than one copy would exist per cell. These molecules probably have arisen by the occasional breakdown of the larger plasmid, a phenomenon observed for some R-factors (17).

DNA homologies among *Agrobacterium* plasmids. When labeled plasmid from *A. tumefaciens* A6 and C-58 was allowed to reassociate with the unlabeled plasmid from various *Agrobacterium* strains, it was found that plasmid homologies varied over a wide range. The most significant conclusion to be drawn from these homology data is that some large *Agrobacter-*

TABLE 2. *Properties of pathogenic Agrobacterium strains*

Strain	Plasmid size ± one standard deviation (megadaltons)[a]	Guanidine utilization[b]		Percentage of plasmid homology with:[c]		Percentage of chromosomal homology with:		Biotype (reference)
		Octopine	Nopaline	A6	C58	A6	NT1	
Agrobacterium tumefaciens								
27	127 ± 4.6 (10)							
	153 ± 9.9 (5)	+	+	15,30	81,88	16	19	2 (19)
223	129 ± 5.7 (5)	+	+	13,12,20	67,67,71	17	20	2 (10)
0362	30 ± 1.2 (7)							
	149 ± 3.7 (6)	−	+	14,17	89,97	31	33	Exceptional[d]
IIBV7	118 ± 4.1 (5)	−	+	27,29	25,28	81	56	1[e]
C-58	120[f]	−	+	8,17	100,100	53	100	1[e]
181	114 ± 2.7 (7)							
	158 ± 4.3 (3)	−	+	12,13	67,76	93	54	Exceptional[d]
A197	158 ± 6.2 (6)	−	+	27	93	ND[g]	ND[g]	1[h]
A6	113 ± 7.0 (4)	+	−	100,100	10,18	100	47	1[e]
CGIC	47 ± 3.1 (6)							
	115 ± 7.4 (4)	+	−	74,86	4.9	58	55	1[i]
15955	118 ± 1.7 (9)	+	−	91,93	3,20	100	43	1[i]
B2A	107 ± 8.3 (8)	+	−	100	16	60	54	1[i]
B6-806	125 ± 8.0 (5)	+	−	87,91	16,16	52	56	1[e]
AT-1	25 ± 1.2 (5)							
	148 ± 6.1 (4)	−	−	11,27	10,19	88	51	1[i]
AT-4	25 ± 0.9 (8)							
	157 ± 5.3 (4)	−	−	18,27	11,19	86	51	1[i]
A. rhizogenes								
TR7	98 ± 3.6 (9)							
	140 ± 4.7 (3)	+	−	5,8	12,20	16	16	2 (10)
A. rubi								
13335	138 ± 3.5 (6)	−	+	6	95	28	18	2 (10)

[a] The number in parentheses is the number of plasmid molecules measured.

[b] Determined radiometrically as previously described (27).

[c] Multiple entries show the homology results obtained with different unlabeled plasmid preparations.

[d] Kersters and DeLey (13) place *A. tumefaciens* 0362 in cluster 1 which corresponds to biotype 1 of Keane et al. (10). This strain is exceptional because it is 3-ketolactose negative, a property usually associated with biotype 2 organisms. Strain 181 is also 3-ketolactose negative but grows well on Schroth's agar which is selective for biotype 1 organisms (22).

[e] Kerr, personal communication.

[f] Watson et al. (27).

[g] ND, Not determined.

[h] Derivative of *A. tumefaciens* C-58, a biotype 1 strain.

[i] Judged to be biotype 1 strains on the basis of a positive 3-ketolactose test (2).

ium plasmids possess little DNA homology with other large *Agrobacterium* plasmids. For example, labeled plasmid from *A. tumefaciens* C-58 has 10 to 18% of its DNA base sequences in common wth the unlabeled plasmid from a strain such as *A. tumefaciens* A6. Because these two plasmids have approximately the same molecular weight (Table 2) they should show the same percentage homology regardless of which plasmid is used as the labeled probe. Indeed, labeled plasmid from *A. tumefaciens* A6 has 8 to 17% of its DNA base sequences in common with unlabeled plasmid from *A. tumefaciens* C-58.

Most of the *Agrobacterium* plasmids examined fall into two genetically distinct groups (Table 2). Plasmids of one group show a high degree of DNA homology with the plasmid from *A. tumefciens* A6. Plasmids of the second group show a high degree of DNA homology to plasmid from *A. tumefaciens* C-58. Plasmid homology within the first group is 74 to 100%, whereas homology between plasmids of the second group is 58 to 97%. Between plasmids of different groups, homology is 30% or less.

Plasmids from *A. tumefaciens* IIBV7, AT-1, AT-4, and *A. rhizogenes* TR7 did not show a high degree of DNA homology with the plasmid

of either *A. tumefaciens* A6 or C-58. To determine whether these plasmids might represent a third genetically distinct group of *Agrobacterium* plasmids, hybridization measurements were made between labeled plasmid from *A. tumefaciens* IIBV7 and unlabeled plasmid from the other bacterial strains. The labeled plasmid from *A. tumefaciens* IIBV7 was found to possess less than 25% DNA homology with the unlabeled plasmid from *A. tumefaciens* AT-1 and AT-4 and *A. rhizogenes* TR7 (data not shown). This indicates that the plasmids from these strains are genetically distinct from the IIBV7 plasmid.

DNA homologies between plasmids and total bacterial DNA. The fact that large plasmids from some *Agrobacterium* strains have little DNA in common raised the possibility that DNA sequences which are plasmid-borne in some strains may actually be present in the chromosomes of other bacterial strains. This possibility was tested by hybridizing labeled C-58 and A6 plasmids with the total bacterial DNA from the strains listed in Table 2. Essentially identical hybridization results were obtained by using either unlabeled total bacterial or unlabeled plasmid. Therefore, we conclude that in cases where low-plasmid homologies are measured, the plasmid of one strain is not present in the chromosome of the other.

DNA homologies between two large plasmids present in the same strain. The presence of two large plasmids in some *Agrobacterium* strains raises some provocative questions concerning the role of large plasmids in pathogenicity. Perhaps only one of these large plasmids is required for pathogenicity, but it is possible that these large plasmids can determine pathogenicity independently of each other. A third possibility is that both plasmids must be present in the same cell in order for the strain to be pathogenic.

To distinguish between these possibilities, the following experiment was done. Plasmid in *A. tumefaciens* 27 was transferred to a plasmidless recipient as previously described (27). The converted recipient strain, A-197, was found to possess a plasmid of 158 megadaltons (Table 2). This value is very close to the mean value of 153 megadaltons that was reported for the larger of the two plasmids in the strain 27. Two other independently isolated recombinants from this cross also contained a large plasmid of approximately 150 megadaltons (data not shown). Because all of these converted strains were isolated from the same cross, they could constitute a clone. Although pathogenic strains that contained the 127 megadalton plasmid of *A. tumefaciens* 27 were not isolated in this experiment, it is still possible that this smaller plasmid can also determine pathogenicity. Nevertheless, these data show that the 153 megadalton plasmid of strain 27 can determine pathogenicity in the absence of the 127 megadalton plasmid.

Because the 127 megadalton plasmid has not been separated from the 153 megadalton plasmid, a direct measurement of the percentage of DNA homology between these two plasmids is not possible. Nevertheless, an estimate of the percentage of DNA homology between these two plasmids was obtained by hybridizing labeled plasmid from strain 27 with the unlabeled 158 megadalton plasmid from strain A197. This hybridization showed that approximately 40% of the plasmid sequences that were present in the plasmid preparation from strain 27 are also present in the plasmid preparation from strain A197 (data not shown). If strain 27 contains the same number of copies of the large and the small plasmids and neither plasmid is selectively lost during isolation, then the 153 megadalton plasmid would represent 54.6% {[153/(152 + 127)] × 100} of the labeled plasmid in strain 27. This calculated value is close to the 40% duplex formation observed between labeled 27 plasmid and unlabeled A197 plasmid. We conclude that the 127 megadalton plasmid possesses little DNA homology with the 153 megadalton plasmid. Because these two large plasmids coexist in the same bacterial cell, they are by definition in different compatibility groups. Apparently in *Agrobacterium* as in the enterics there is a lack of homology between plasmids of different compatibility groups (7, 8). Furthermore, the low degree of DNA homology between labeled A6 plasmid and unlabeled plasmid from *A. tumefaciens* 27 shows that the 127 megadalton plasmid of *A. tumefaciens* 27 has little homology with A6 plasmid.

Determination of chromosomal DNA homologies. Chromosomal DNA homologies were measured by using A6 and NT1 total bacterial DNA which was labeled in vitro (specific activity $>5 \times 10^6$ counts/min per μg). Because strain NT1 does not contain a plasmid, its DNA is a better probe for determining chromosomal DNA homologies than the total bacterial DNA from the plasmid-containing parent strain C-58. We have found that total *A. tumefaciens* bacterial DNA contains about 3% plasmid (4, 27). Therefore, the maximum percentage of duplex formation possible between the labeled plasmid from *A. tumefaciens* A6 and the unlabeled bacterial DNA would be approximately 3%, a negligible contribution. Reassociation of radioactively labeled chromosomal DNA from

A. tumefaciens NT1 and A6 with unlabeled bacterial DNA from the strains used in this study revealed that the chromosomal DNA homologies vary over a wide range (Table 2); 16 to 100% DNA homology with *A. tumefaciens* A6, and 18 to 56% DNA homology with *A. tumefaciens* NT1. Low DNA homology between virulent strains of *Agrobactrium* has been previously reported (6).

DISCUSSION

The data presented strengthen and extended the original observation (28) of the absolute correlation between the presence of a large plasmid and pathogenicity in *Agrobacterium*. All pathogenic strains in the present study isolated in several parts of the world, 12 of which were not studied previously (28), contain a large plasmid. Our assumption that a large plasmid is required for pathogenicity is based on the following considerations. First, the plasmid in strain C-58 has been shown to be necessary for pathogeniciy because the loss of plasmid sequences results in a simultaneous loss of pathogenicity (27), and reintroduction of the plasmid results in the simultaneous gain of pathogenicity (26, 27). Second, the transfer of a large plasmid from strains 223, 27, 0362, and EU6 to a nonpathogenic strain resulted in the strain becoming pathogenic (25–27 and unpublished data). Third, Kerr (11, 12) has shown that pathogenicity can be transferred from a large number of donor strains to nonpathogenic strains. Although he did not demonstrate plasmid transfer in any of these experiments, it was subsequently shown in several cases employing the same donor and recipient strains that plasmid transfer did occur concomitantly with transfer of pathogenicity (26, 27, and unpublished data). Fourth, strains IIBV7 and IIBNV6, isolated by Armin Braun in the course of passaging *A. tumefaciens* IIB, appear to differ only in pathogenicity (27). Strain IIBV7 is pathogenic and contains a plasmid and strain IIBNV6 is nonpathogenic and does not contain a plasmid. Thus the large plasmid in strain IIBV7 appears to be required for pathogenicity. From all of these considerations we believe our assumption, that a large plasmid is required for pathogenicity, is reasonable. However, it is also reasonable to expect that chromosomal genes are important for tumor induction. For this reason, we refer to the plasmid required for tumor induction simply as a large plasmid. At this time, such a vague description is appropriate because it has not yet been shown that all large *Agrobacterium* plasmids are required for tumor induction, nor do these data suggest that plasmid genes are incorporated into the tumor DNA or that only plasmid genes are necessary for tumor induction.

The plasmid hybridization data presented in this paper allow an estimation of the extent of DNA homology between the most distantly related large *Agrobacterium* plasmids. The plasmids of *A. tumefaciens* C-58 and CGIC in one case exhibit only 5% homology. If this value is corrected for the self-renaturation of the labeled probe, which was about 3% in this case, then about 2% homology exists between these two plasmids. Because each of these plasmids is approximately 100 megadaltons in size, they possess 2 megadaltons of common DNA sequences, sufficient to code for two to four average-sized proteins. These common genes may represent functions essential for pathogenicity. If so, one would predict that the same homologous DNA region would be found on all large *Agrobacterium* plasmids. A direct test for common genes on all large *Agrobacterium* plasmids will require isolation of these DNA sequences. If a common small DNA region can be demonstrated on all large *Agrobacterium* plasmids, it will be tempting to suppose that it codes for functions that are concerned with tumor induction. The alternative possibility that these few common plasmid genes code for DNA replication or other functions necessary for plasmid maintenance must still be considered.

The finding that large plasmids from some pathogenic *Agrobacterium* strains have little DNA in common makes it unlikely that all large *Agrobacterium* plasmids evolved from a common large plasmid. Therefore, it would not be surprising that such distantly related plasmids could coexist in the same bacterial cell. Because pathogenicity is associated with the presence of a very large plasmid, a high degee of DNA homology between all large *Agrobacterium* plasmids might have been predicted. The data presented here show that this clearly is not the case. The issue now raised is the mystery of why these plasmids invariably seem to be so large. If only two to four genes deal with tumor induction, as the extent of homology between distantly related plasmids might suggest, the role of the remainder of the plasmid genome is not clear. The fact that smaller plasmids, which are required for pathogenicity, have not been discovered suggests that either the physical size of the plasmid or the unknown (and apparently varied) functions for which it codes have an imporant bearing on the course of tumor induction. If the small region of homology between distantly related plasmids does indeed code for tumor induction functions, it is

possible that this small DNA fragment is the long sought "tumor-inducing principle." Although the entire plasmid genome does not appear to be transmitted to tumor cells in the transformation process (5), hybridization studies thus far do not rule out the possible presence of a small specific fragment of the plasmid genome in the transformed tissue. Recent findings with transformed rat cells show that adenovirus 2 inserts a specific fragment of its genome into the host (24). It is essential that this possibility be tested in this plant transformation system before the question of foreign DNA in crown gall tumor cells can be settled.

A close correlation has been reported by Lippincott et al. (15) between pathogenicity in *Agrobacterium* strains and their ability to utilize the unusual amino acids octopine [N^2-(D-1-carboxyethyl)-L-arginine] and/or nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine]. A comparison of octopine and nopaline utilization traits with plasmid homology data (Table 2) reveals an intriguing correlation. All bacterial strains that contain plasmids homologous to that of A6, an octopine-utilizing strain, are themselves octopine utilizers. Similarly, all bacterial strains whose plasmids are closely related to that of C-58, a nopaline utilizer, can themselves utilize nopaline. Therefore, it would not be surprising if octopine and nopaline utilization traits are determined by plasmid genes. The ability to utilize nopaline is indeed a plasmid-borne trait in strain C-58 (21, 25, 27). It is probably also a plasmid-borne trait in strains 27 (27) and 223 (unpublished data), because transfer of pathogenicity by the procedure of Kerr (11), which is mediated by plasmid transfer (26, 27), results in transfer of the ability to utilize nopaline. Since the genes for octopine utilization are also present in *A. tumefacians* 27 and 223 but are not transferred by this procedure, it is not clear whether octopine utilization is a plasmid-borne marker in these strains where a large plasmid probably carries the genes for nopaline utilization. However, in *A. tumefaciens* NCPPB 1001, the abiliy to utilize octopine is probably a plasmid-borne trait, for it has been transferred wth pathogenicity to a nonpathogenic recipient bacterial strain (Kerr, personal communication). The finding that plasmids from most octopine-utilizing strains are closely related (Table 2) suggests that in these strains the ability to utilize octopine may be a plasmid-borne trait.

Although plasmids from the majority of *Agrobacterium* strains exhibit a high degree of homology with either the A6 or C-58 plasmid, there are exceptions. Plasmids from several strains examined in this study do not appear to be closely related to either group. One such exceptional strain, *A. rhizogenes* TR7, utilizes octopine, another strain, *A. tumefaciens* IIBV7, utilizes nopaline, and *A. tumefaciens* AT-1 and AT-4 utilize neither octopine nor nopaline. In addition, the 127 megadalton plasmid from strain 27 is unrelated to either the A6 or the C-58 plasmid. This plasmid possibly carries genes for the utilization of octopine. It is clear that the octopine/nopaline utilization pattern cannot be used to predict plasmid homology groups with absolute certainty, although members of a given homology group appear to share utilization patterns.

Even though the large plasmid from *A. tumefaciens* IIBV7 does not show a high degree of homology to either the A6 or the C-58 plasmid, direct evidence suggests that this large plasmid is required for tumor induction and that it carries the gene(s) which codes for nopaline utilization. The nonpathogenic strain IIBNV6 appears to be an isogenic derivative of strain IIBV7. The lack of pathogenicity in strain IIBNV6 and its inability to utilize nopaline appear to be due to the lack of the plasmid sequences which are present in strain IIBV7 (27). Therefore, the lack of homology between some large *Agrobacterium* plasmids does not argue against a positive correlation between large plasmids and pathogenicity. Neither does this lack of homology exclude the possibility that these large plasmids carry the genes for octopine or nopaline utilization. Direct evidence is not yet available to suggest that the large plasmids in *A. tumefaciens* AT-1 and AT-4 and *A. rhizogenes* TR7 are required for tumor induction or that the plasmid from strain TR7 carries the gene(s) for octopine utilization. However, there is no reason to assume otherwise. The 127 megadalton plasmid of strain 27 also possesses a low degree of homology to both the A6 and the C-58 plasmids. Whether this plasmid is required for tumor induction is questionable because this strain contains another large plasmid that is required for pathogenicity when transferred to a nonpathogenic, plasmidless strain.

Based on a numerical analysis of a large number of characters, Keane et al. (10) have shown that pathogenic strains of *Agrobacterium* can be placed in two biotypes. With two exceptions, pathogenic biotype 1 organisms are classified as *A. tumefaciens* whereas pathogenic biotype 2 organisms may be classified as *A. rubi*, *A. rhizogenes*, or *A. tumefaciens*. If such a numerical classification has a genetic basis, then the chromosomal DNA homologies

of bacterial strains within a particular biotype would be expected to be greater than the chromosomal DNA homologies of bacterial strains from different biotypes. The chromosomal homology data presented in Table 2 are consistent with this prediction. In general, the DNA homologies between biotype 2 organisms and labeled probe DNA from biotype 1 organisms were low. Interbiotype percentage of chromosomal homology ranged from 16 to 22% with *A. tumefaciens* A6 as a labeled probe, and from 19 to 23% with *A. tumefaciens* NT1 as a labeled probe. Conversely, all biotype 1 organisms possess at least 55% DNA homology with one or the other of the labeled probes, both of which were isolated from biotype 1 strains. The lowest percentage of chromosomal DNA homology observed between two biotype 1 organisms (NT1 and 15955) was 43%. It is clear from this limited taxonomic analysis and from more extensive studies of DeLey (6) that a numerical classification of *Agrobacterium* reflects genetic similarities more closely than the traditional species classification which is based mainly on pathogenicity and host range.

Comparison of chromosomal DNA homology data with the plasmid homology data clearly shows that plasmid homology between two bacterial strains can be either higher or lower than the chromosomal DNA homology. For example, the chromosomal DNA of *A. tumefaciens* 27 shows only 10% homology to that of *A. tumefaciens* C-58, yet these two strains contain plasmid that is 81 to 88% homologous. Conversely, the chromosomal DNA homology between strains IIBV7 and A6 is 81% whereas their plasmids are only 27% related. Further, the plasmids from *A. tumefaciens* AT-1 and AT-4 show low homology to both labeled plasmids but the chromosomal homologies between these strains and the A6 and C-58 chromosomes are high. Thus, the plasmid homology data do not correlate with either a genetic classification (chromosomal DNA homology data) or a phenotypic classification (numerical analysis of Kerr) of *Agrobacterium* strains.

ACKNOWLEDGMENTS

We thank M.-D. Chilton, M. P. Gordon, and A. J. Bendich for their help in preparation of this manuscript, as well as for many stimulating discussions. We are grateful to Alice Montoya, Stephanie Lara, and Frank White for excellent technical assistance.

T. C. was a predoctoral trainee supported by Public Health Service grant 5T01GM00511 from the National Institute of General Medical Sciences. These studies were also supported by Public Health Service grant CA-13015 from National Cancer Institute.

LITERATURE CITED

1. **Beardsley, R. E.** 1955. Phage production by crown-gall bacteria and the formation of plant tumors. Am. Nat. 89:175-176.
2. **Bernaerts, M. J., and J. DeLey.** 1963. A biochemical test for crown gall bacteria. Nature (London) 197: 406-407.
3. **Braun, A. C.** 1947. Thermal studies on the factors responsible for tumor initiation in crown gall. Am. J. Bot. 34:234-240.
4. **Chilton, M.-D., T. C. Currier, S. K. Farrand, A. J. Bendich, M. P. Gordon, and E. W. Nester.** 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. U.S.A. 71:3672-3676.
5. **Chilton, M.-D., S. K. Farrand, F. Eden, T. C. Currier, A. J. Bendich, M. P. Gordon, and E. W. Nester.** 1975. Is there foreign DNA in crown gall tumor DNA?, p. 297-311. *In* R. Markham, D. R. Davies, D. A. Hopwood, and R. W. Horne (ed.), Second annual John Innes symposium. North-Holland Publishing Company, Amsterdam.
6. **DeLey, J.** 1974. Phylogeny of procaryotes. Taxon 23:291-300.
7. **Grindley, N. D. F., G. O. Humphreys, and E. S. Anderson.** 1973. Molecular studies of R factor compatibility groups. J. Bacteriol. 115:387-398.
8. **Guerry, P., and S. Falkow.** 1971. Polynucleotide sequence relationships among some bacterial plasmids. J. Bacteriol. 107:372-374.
9. **Hamilton, R. H., and M. Z. Fall.** 1971. The loss of tumor-initiating ability in *Agrobacterium tumefaciens* by incubation at high temperature. Experimentia 27:229-230.
10. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23:585-595.
11. **Kerr, A.** 1969. Transfer of virulence between strains of *Agrobacterium*. Nature (London) 223:1175-1176.
12. **Kerr, A.** 1971. Acquisition of virulence by non-pathogenic isolates of *Agrobacterium radiobacter*. Physiol. Plant Pathol. 1:241-246.
13. **Kersters, K., J. DeLey, P. H. A. Sneath, and M. Suckin.** 1973. Numerical taxonomic analysis of *Agrobacterium*. J. Gen. Microbiol. 78:227-239.
14. **Kleinschmidt, A. K., D. Lang, D. Jaeherts, and R. K. Zahn.** 1962. Preparation and length measurements of the total deoxyribonucleic acid content of T2 bacteriophages. Biochim. Biophys. Acta 61:857-867.
15. **Lippincott, J. A., R. Beiderbeck, and B. B. Lippincott.** 1973. Utilization of octopine and nopaline by *Agrobacterium*. J. Bacteriol. 116:378-383.
16. **Marmur, J.** 1961. A procedure for isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 12:468-487.
17. **Nisioka, T., M. Michiko, and R. Clowes.** 1969. Composite circular forms of R-factor deoxyribonucleic acid molecules. J. Bacteriol. 97:376-385.
18. **Riker, A. J., W. M. Banfield, W. H. Wright, G. W. Keitt, and H. E. Sagen.** 1930. Studies on infectious hairy root of nursery apple trees. J. Agric. Res. 41:507-540.
19. **Roberts, W. P., and A. Kerr.** 1974. Crown gall induction: serological reactions, isozyme patterns and sensitivity to mitomycin C and to bacteriocin, of pathogenic and non-pathogenic strains of *Agrobacterium radiobacter*. Physiol. Plant Pathol. 4:81-91.
20. **Schachat, F. H., and D. S. Hogness.** 1973. Repetitive sequences in isolated Thomas circles from *Drosophila melanogaster*. Cold Spring Harbor Symp. Quant. Biol. 38:371-381.
21. **Schilperoort, R. A., J. J. M. Dons, and H. Ras.** 1975. Characterization of the complex formed between PS8 cRNA and DNA isolated from A6-induced sterile

crown gall tissue, p. 253-268. *In* R. Markham, D. R. Davies, D. A. Hopwood, and R. W. Horne (ed.), Second annual John Innes symposium. North-Holland Publishing Company, Amsterdam.

22. **Schroth, M. N., J. P. Thompson, and D. C. Hildebrand.** 1965. Isolation of *Agrobacterium tumefaciens-A. radiobacter* group from soil. Phytopathology 55:645-647.
23. **Schroth, M. N., A. R. Weinhold, A. H. McCain, D. C. Hildebrand, and N. Ross.** 1971. Biology and control of *Agrobacterium tumefaciens*. Hilgardia 40:539-552.
24. **Sharp, P. A., U. Pettersson, and J. Sambrook.** 1974. Viral DNA in transformed cells. I. A study of the sequences of adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. 86:709-726.
25. **Van Larebeke, N., G. Engler, M. Holsters, S. Van den Elsacker, I. Zaenen, R. A. Schilperoort, and J. Schell.** 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown-gall inducing ability. Nature (London) 252:169-170.
26. **Van Larebeke, N., Ch. Genetello, J. Schell, R. A. Schilperoort, A. K. Hermans, J. P. Hernalsteens, and M. von Montagu.** 1975. Acquisition of tumor-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature (London) 255:742-743.
27. **Watson, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 123:255-264.
28. **Zaenen, I., N. Van Larebeke, H. Teuchy, M. van Montagu, and J. Schell.** 1974. Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J. Mol. Biol. 86:109-127.