JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 76-80

Vol. 129, No. 1
Printed in U.S.A.

Plasmids in Avirulent Strains of *Agrobacterium*

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Received for publication 7 September 1976

Twelve strains of *Agrobacterium radiobacter* isolated from naturally occurring crown galls or soil were found to be avirulent on sunflower, tomato, *Kalanchoe*, and carrot. Eleven strains contained plasmids of molecular weights 77×10^6 to 182×10^6 as determined by electron microscopy. One strain contained only a smaller plasmid (50×10^6 daltons). Several strains had both large and small (ca. 11×10^6 daltons) plasmids; one strain contained two large plasmids (112×10^6 and 136×10^6 daltons). Hybridization reactions of virulence plasmids from *Agrobacterium tumefaciens* strains C58 and A6 with plasmids from each of the *A. radiobacter* strains revealed that some *A. radiobacter* plasmids had less than 10% homology to either the C58 or A6 plasmids. Plasmids from some strains had approximately 50% homology with the C58 plasmid, but only one *A. radiobacter* plasmid contained more than 10% homology to the A6 plasmid. The presence of large plasmids in *A. radiobacter* strains did not correlate with sensitivity to agrocin 84; however, the utilization of the amino acid derivatives octopine and nopaline was generally correlated to partial base sequence homology to the C58 plasmid. We conclude that all large plasmids found in *Agrobacterium* strains are not virulence associated, although they may share base sequence homology with a virulence-associated plasmid. Further, plasmids from tumorigenic strains may be more closely related by base sequence homology to plasmids from nonpathogenic strains than to plasmids from other pathogenic strains.

In a survey of strains of *Agrobacterium*, causal agent of crown gall disease, Zaenen et al. (18) found large plasmids (molecular weight, 96×10^6 to 156×10^6) in 11 pathogenic strains, but no plasmid of any size was reported in any of 8 nonpathogenic strains examined. The hypothesis was tendered that the large plasmids carried the genetic information for the "tumor inducing principle" proposed by Braun (5). Other workers have reported genetic evidence for plasmid-borne virulence genes. Loss of pathogenicity of the virulent strain C58 of *Agrobacterium tumefaciens* occurred concomitantly with the loss of a large plasmid (15, 17). Moreover, the introduction of the virulence-associated plasmid into an avirulent plasmidless strain always confers virulence to the recipient strain (16, 17; M.-D. Chilton, S. K. Farrand, R. Levin, and E. W. Nester, Genetics, in press; M. P. Gordon, S. K. Farrand, D. Sciaky, A. Montoya, M.-D. Chilton, D. Merlo, and E. W. Nester, *In* I. Rubenstein, ed., *Proceedings of a Symposium on the Molecular Biology of Plants*, in press; E. W. Nester, M. Drummond, M.-D. Chilton, D. Merlo, A. Montoya, D. Sciaky, and M. P. Gordon, *In* F. Young, ed., *Tenth Annual Miles Symposium: Impact of Recombinant Molecules on Science and Society*, in press).

The metabolism of two unusual amino acid derivatives, octopine [N^2-(D-1-carboxyethyl)-L-arginine] and nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine], is also closely correlated with virulence (13) and the presence of large plasmids in pathogenic *Agrobacterium* strains (4, 17). Generally, strains that metabolize one or the other of these guanido amino acids incite tumors which contain high amounts of the same amino acid (compared to undetectable amounts in healthy plants). The gene controlling octopine and nopaline production by tumors is plasmid borne (4; Gordon et al., *In* I. Rubenstein, ed., *Proceedings of a Symposium on the Molecular Biology of Plants*, in press; Nester et al., *In* F. Young, ed., *Tenth Annual Miles Symposium: Impact of Recombinant Molecules on Science and Society*, in press).

The purpose of the present study was to confirm the expectation that naturally occurring nonpathogenic strains do not contain large plasmids and to screen these strains for biochemical properties known to be associated with the plasmid in virulent strains. We anticipated that such an approach might elucidate

the relationships between pathogenic and nonpathogenic strains and provide additional insight into the role of the plasmid in tumor induction.

(Portions of this work were presented at the University of Minnesota Symposium on the Molecular Biology of Plants, March 1976, and at the 68th Annual Meeting of the American Phytopathological Society, Kansas City, Mo., in July 1976.)

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains employed and their sources are listed in Table 1. All *Agrobacterium radiobacter* strains except strains RV_3, 6467, and 84 were screened for virulence on tomato plants and classified as to biotype on the basis of 14 diagnostic tests (10) by L. Moore, Oregon State University. The pathogenic *A. tumefaciens* strains A6 and C58 have been described previously (7). Stock cultures established from single colonies were isolated and maintained on nutrient agar. All subsequent manipulations were performed on cultures derived from single colonies. Cells were grown for deoxyribonucleic acid (DNA) extraction in mannitol-glutamate medium containing 0.025% yeast extract as previously described (17).

Pathogenicity assays. All the *A. radiobacter* strains were nonpathogenic on the four plant hosts tested. Stems of sunflower (*Helianthus annuus* Mammoth variety) and tomato (*Lycopersicon esculentum*, several varieties) seedlings were wound inoculated with a sterile toothpick which had been touched to a colony of the test strain. *Kalanchoe daigremontiana* leaves and carrot disks (*Daucus carota*, purchased from commercial food markets) were inoculated as previously described (17) or by the method of Ark and Schroth (2). Virulence was scored at 7, 14, and 21 days after inoculation. In some carrot disk assays, strain AB2/72 induced small overgrowths that did not appear with the same frequency or vigor as those induced by pathogenic strains.

Biochemical tests. Ability of the bacteria to utilize radioactively labeled octopine or nopaline was assayed as described by Watson et al. (17). Production of 3-ketolactose was assayed by the method of Bernaerts and DeLey (3).

Sensitivity of the strains to growth inhibition by agrocin 84 produced by strain 84 (9) was assayed on AB minimal medium (6) by slight modifications of the method previously described (17).

Preparation of plasmid and total DNA. Covalently closed circular DNA of molecular weight up to 182×10^6 was prepared by the method of Currier and Nester (Anal. Biochem., in press). The DNA was stored at 4°C for 15 to 30 days to allow conversion to the open circular form, after which the electron microscopic procedures of Kleinschmidt et al. (11) and Lang (12) were employed for molecular weight determinations. These techniques routinely allowed visualization of both large and small open circular DNAs from multiple plasmid-containing strains in the same microscopic field.

Total bacterial DNA was prepared using a modification of the Marmur procedure (14).

Plasmid DNA homology. Procedures for hybridization of high (driver) concentrations of unlabeled

TABLE 1. *Source and description of A. radiobacter strains*

Strain	Natural source	Pathogenicity[a]	Biotype[b]	Guanido amino acid utilization		Sensitivity to bacteriocin 84
				Octopine	Nopaline	
RV_3[c]	No information available	−	1	−	−	−
6467[d]	Soil isolate	−	1	−	−	−
AB11/73[e]	*Lippia canescens*	−	1	−	−	+
E13/73[e]	*Dahlia* spp.	−	1	−	−	−
S11/72A[e]	Uncertain	−	1	−	−	+
T24/75[e]	*Rosa* spp.	−	1	−	+	−
T20/73[e]	*Rosa* spp.	−	1	±	+	−
1[e]	*Prunus cerasifera*	−	1	±	+	+
3[e]	*Prunus persica*	−	1	±	+	−
G12/73[e]	*Prunus persica*	−	1	+	+	+
AB2/72[e]	*Lippia canescens*	−[f]	2	+	−	+
84[g]	Soil around peach gall	−	2	+	+	
C58[h]		+	1	±	+	+
A6[h]		+	1	+	−	−

[a] Scored on sunflower, tomato, carrot, and *Kalanchóe daigremontiana*.
[b] Biotypes described by Keane et al. (10).
[c] Obtained from J. Lippincott, Northwestern University, Evanston, Ill.
[d] Obtained from American Type Culture Collection.
[e] Obtained from L. Moore, Oregon State University, Corvallis, Ore.
[f] Variable reaction on carrot disks.
[g] Obtained from A. Kerr, University of Adelaide, Glen Osmond, Australia.
[h] *A. tumefaciens* strains described previously (7).

A. radiobacter plasmids with labeled plasmid probe from pathogenic strains C58 and A6 were as described previously (6, 7). Plasmids from strains C58 and A6 were chosen as probes because they represent the two major types of virulence-associated plasmids, sharing only about 14% common sequences (8).

The percentage of duplex formation was corrected for reassociation of the probe DNA alone (1 to 5%) and then normalized to control reactions by dividing by the percentage of duplex formation (40 to 60%) attained in the corresponding homologous control reaction (i.e., radioactive probe plus unlabeled driver plasmid isolated from strain A6 or C58). In cases where the test strain contained a sole plasmid which was smaller than the probe plasmid (as measured by electron microscopy), the maximum degree of homology expected was less than 100%. Accordingly, the observed extent of duplex formation (corrected as above) was normalized to 100% homology by dividing that value by the ratio of the size of the test plasmid to the size of the labeled probe plasmid. In this way we derived a better estimate of the relatedness of the amount of genetic information in the smaller plasmid to the DNA of the larger probe plasmids.

Some *A. radiobacter* strains contained multiple plasmids. In these cases, normalization of percentage of duplex formation was not possible, since the absolute size of the total test plasmid genome is not easily known.

RESULTS

Presence of plasmids. All nonpathogenic strains had at least one plasmid whose molecular weight was greater than 50×10^6 (Table 2, columns A and B). Several strains had more than one plasmid, usually a large (70×10^6 to 130×10^6 daltons) and a small plasmid (ca. 11×10^6 daltons). Strain E13/73 contained two large plasmids (136×10^6 and 111×10^6 daltons). Strain RV_3 contained the largest *Agrobacterium* plasmid we have observed (182×10^6 daltons).

Strain AB2/72 yielded three plasmids, the two smaller of which differed significantly in size as judged by the Student's *t* test. To our knowledge, this is the first report of more than two plasmids in an *Agrobacterium* strain.

Plasmid homologies. Plasmids prepared from several nonpathogenic strains had significant homology with the plasmid from a pathogenic strain, C58 (Table 2, column D). In cases

TABLE 2. *Properties of A. radiobacter plasmids*

Strain	(A) No. of plasmid size classes	(B) Plasmid mol wt ($\times 10^{-6}$)[a]	(C) Daltons of test plasmid/daltons of probe plasmid		(D) Plasmid DNA homology as % of control[a]		(E) Normalized plasmid homology	
			C58 plasmid	A6 plasmid	With C58 plasmid	With A6 plasmid	With C58 plasmid	With A6 plasmid
Nonpathogens								
Biotype 1								
RV_3	1	182.1 ± 5.4[b] (5)	1.52	1.61	8 ± 1[b] (2)	5 (2)	8	5
6467	1	50.4 ± 1.4 (9)	0.42	0.45	13 ± 5(3)	24 ± 6 (3)	31	53
T24/75	1	78.6 ± 1.9 (11)	0.66	0.70	29 ± 1 (2)	2 (2)	44	3
T20/73	1	77.6 ± 2.3 (10)	0.65	0.69	27 ± 2 (2)	1 (1)	42	1
1	1	92.4 ± 2.0 (6)	0.77	0.82	46 ± 5 (2)	3 (1)	60	4
3	1	115.6 ± 2.6 (4)	0.96	1.02	51 ± 2 (2)	1 (2)	53	1
AB11/73	2	124.8 ± 4.1 (4) 10.6 ± 0.6 (28)	-	-	5 (2)	2 (2)	-	-
E13/73	2	135.8 ± 6.1 (4) 111.7 ± 7.5 (8)	-	-	5 (4)	4 (2)	-	-
S11/72A	2	126.7 ± 9.3 (6) 10.9 ± 0.7 (112)	-	-	9 ± 2 (2)	3 (1)	-	-
G12/73	2	99.2 ± 7.6 (10 12.1 ± 0.5 (30)	-	-	38 ± 5 (2)	2 (2)	-	-
Biotype 2								
84	2	124.0 ± 4.0 (5) 29.8 ± 1.6 (23)	1.28	1.36	35 ± 7 (9)	3(3)	-	-
AB2/72	3	90.9 ± 3.0 (9) 31.1 ± 0.9 (11) 23.3 ± 1.0 (13)	-	-	9 ± 1 (2)	1 (1)	-	-
Pathogens								
C58	1	120 ± 4.5[c] (9)	1.00	1.06	100	14[d]	100	14
A6	1	113 ± 7.0[d] (4)	0.94	1.00	14[d]	100	15	100

[a] The number in parentheses indicates the number of individual measurements.
[b] Standard deviation. Homology values of 5% or less are not greater than experimental error.
[c] Watson et al. (17).
[d] Currier and Nester (7).

where the size of the test plasmid was considerably smaller than that of the probe plasmid, a relatively low degree of measured homology indicated a significant degree of actual homology. For example, the size of the plasmid of strain 6467 (50.4×10^6 daltons) is 42% of the C58 plasmid (120×10^6 daltons; Table 2, columns B and C). An excess of 6467 plasmid DNA duplexed with 13% of the radioactive C58 plasmid (Table 2, column D). Since 42% of labeled C58 plasmid would be the maximum amount with which the 6467 plasmid could react, the 13% measured homology actually indicates that 31% ([13 ÷ 42] × 100) of the 6467 plasmid base sequences are homologous to those of the C58 plasmid (Table 2, column E).

When similar adjustments were made to the data obtained from the other strains that contained only a single plasmid, we found that plasmids from five of the six strains had from 30 to 60% base sequence homology to the C58 plasmid. Only one strain, 6467, contained a plasmid with significant homology to the A6 plasmid. These normalized homology values in column E represent the percentage of homology expected if one were to hybridize radioactive plasmid from the nonpathogenic isolate and unlabeled plasmid DNA from the pathogenic strains. However, these values possess a considerable degree of statistical error due to the cumulative errors in the determination of the plasmid sizes and measurement of duplex formation, and so serve only as an estimation of the opposite reactions.

Plasmid preparations from four of the six strains which contained multiple plasmids contained very little total sequence homology to either the C58 or A6 plasmid. Total plasmid from the other two strains, G12/73 and 84, duplexed with 38 and 35%, respectively, of the C58 plasmid (Table 2, column D). Since it is not clear which plasmid is hybridizing to the C58 plasmid probe, these data are difficult to interpret.

Hybridization reactions were also performed between total bacterial DNA from the test strains and the radioactive plasmid probes. The extent of homology found between probe and total DNA was essentially the same as that detected between probe and plasmid DNA in all cases (data not shown). These results indicate that large amounts of sequences homologous to the probe plasmid were not present in the bacterial chromosomes. Currier and Nester (7) also did not detect any virulence-associated plasmid sequences in the chromosomes of virulent *A. tumefaciens* strains.

Utilization of octopine and nopaline. Five of the biotype 1 strains did not metabolize either exogenous octopine or nopaline (Table 1) during 7 h of incubation. The other five biotype 1 strains preferentially metabolized nopaline in all cases but one, G12/73, which utilized octopine and nopaline equally well (indicated by + in Table 1). The other nopaline utilizers degraded octopine at a decreased rate (approximately 10% as well as strains which utilized octopine exclusively; indicated by ±). One strain, T24/75, utilized nopaline exclusively.

Two biotype 2 nonpathogens were examined. Strain 84 utilized octopine and nopaline equally well, whereas strain AB2/72 utilized only octopine.

Agrocin sensitivity. Agrocin 84 affected the growth of several of the nonpathogenic strains (Table 1). In contrast to the results of Engler et al. (9) with other *Agrobacterium* strains, we found no correlation between the presence of large plasmids and agrocin 84 sensitivity in these nonpathogenic strains. Further, the degree of plasmid homology to the C58 plasmid and bacteriocin sensitivity was not correlated.

DISCUSSION

The results of this study show that the inability of *Agrobacterium* strains to incite tumors does not correlate with any of the properties that were examined. To our surprise, it did not even correlate with the absence of large plasmids. This expectation was based on the reported correlation between tumor-inducing ability and the presence of a large plasmid (18). Recently, Lin et al. (Abstr. Am. Phytopathol. Soc. 1976, 82, p. 98) reported the isolation of large plasmids (molecular weight, 150×10^6 to 170×10^6) from 10 *A. radiobacter* strains obtained from various laboratories. Taken together, these results indicate unequivocally that the presence of large plasmids is not a characteristic exclusive to virulent *Agrobacterium* strains.

Zaenen et al. (18) detected no plasmid DNA in strain RV_3 by sedimentation in neutral sucrose gradients. We have detected a very large plasmid in this strain. The discrepancy with our results is undoubtedly due to the different techniques employed to demonstrate the presence of plasmids and the extremely large size of the RV_3 plasmid. In our laboratory, it was difficult to routinely obtain intact, closed circular plasmid DNA from this strain.

Although plasmids from natural isolates of pathogenic *Agrobacterium* strains were found to be homologous to the A6 plasmid in many cases (7), only 1 of the 12 nonpathogenic isolates examined in this study had extensive homology to the A6 plasmid. Since the nonpathogenic strains were isolated from different parts of the

world, it seems remarkable that their plasmids, if homologous to one of the plasmid probes, were always homologous to the C58 plasmid. In addition, the plasmids from some nonpathogenic strains were not significantly homologous to either of the probe plasmids, a result also observed with plasmids from *A. tumefaciens* strains (7). The significance of these observations is not clear.

The functions of *A. radiobacter* plasmids are unknown. In *A. tumefaciens* strains, besides pathogenicity, the large plasmids have been implicated in agrocin 84 sensitivity (9), bacteriophage exclusion (16), and utilization of nopaline (4, 17) or octopine (4). It seems likely that the same functions are also encoded by plasmids in nonpathogenic strains bearing these traits. Indeed, a general correlation is seen in that strains which utilize octopine and/or nopaline have a higher degree of homology to the C58 plasmid than most of the nonutilizing strains. Strains 6467 and AB2/72 are obvious exceptions to the generalization, however. The further observation that plasmids of nonpathogenic strains may be significantly homologous to a plasmid which is known to confer pathogenicity in strain C58 reinforces the earlier finding of Currier and Nester (7) that the bulk of the genetic information encoded in the plasmid DNA may not relate directly to pathogenicity.

The results of this study and those of others (8, 10) reveal that there is no clear distinction between the two species *A. tumefaciens* and *A. radiobacter* other than pathogenicity. Since changes in pathogenicity force the individual strains to cross taxonomic species lines, it seems exceedingly arbitrary to base the species designation on pathogenicity. It seems more reasonable to suggest that virulent and avirulent strains be considered as the same species. A similar suggestion has been made previously (10).

ACKNOWLEDGMENTS

The excellent technical assistance of Alice Montoya and Peter Hartel is gratefully acknowledged. We thank L. Moore, Oregon State University, for generously supplying most of the strains in this study and for providing the information on their diagnostic tests.

D.M. was supported by Public Health Service postdoctoral research fellowship 1 F32 CA05222-01 from the National Cancer Institute. This work was also supported by Public Health Service grant CA-13015 from the National Cancer Institute.

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