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Broad-Host-Range Agrocin of *Agrobacterium tumefaciens*

MAVIS HENDSON,[1] LIZZIE ASKJAER,[1] JENNIFER A. THOMSON,[1]†* AND MARC VAN MONTAGU[2]

Department of Genetics, University of the Witwatersrand, Johannesburg 2001, South Africa,[1] and Laboratoria voor Genetika en voor Histologie, Rijksuniversiteit Gent, B-9000 Gent, Belgium[2]

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Eighteen strains of *Agrobacterium tumefaciens* isolated from crown galls were tested for agrocin production. Of six agrocin-producing strains, one (D286) produced a broad-host-range agrocin active against strains carrying nopaline, octopine, and agropine type Ti plasmids. Sensitivity to agrocin D286 was found to map in the 11- to 18-megadalton region of the nopaline Ti plasmid pTiC58. The agrocin was partially purified, and its physical characteristics were consistent with its being a nucleotide, as is agrocin 84. Agrocin D286 was shown to inhibit DNA, RNA, and protein syntheses. Strain D286 spontaneously lost its pathogenicity, and its potential for use in the biological control of crown gall is discussed.

Successful biological control of crown gall with *Agrobacterium radiobacter* 84, which produces agrocin 84, has been reported in southern Australia and several other countries (1, 4, 8, 10, 11, 13, 17–19). This represents the first commercial use of a specific microorganism to control a plant pathogen in soil, and it is the first commercial use of a bacterium to control any plant disease (11). Only strains of *Agrobacterium tumefaciens* harboring the nopaline Ti plasmid are potentially sensitive to strain 84, but among these are some found to be resistant either in vitro or in vivo (12, 13, 18), and on some hosts strain 84 exhibits biological control of pathogenic strains that are insensitive in vitro (17). This may suggest that other mechanisms, such as competition for infection sites, are utilized by strain 84 to prevent the infection of some host species (15).

In a study on the effect of strain 84 on pathogenic European strains, it failed to control 7 of 14 (18). It also failed to protect field-grown apple seedlings in Washington and rose plants in Texas and Pennsylvania. In Greece, no biotype 3 strain (isolated from grapevines) was subject to biological control by strain 84 (13). In contrast, crown gall has been reduced to less than 1% on commercially produced roses in Australia and New Zealand (18). Having postulated that bacteriocins should exist which are able to kill agrocin 84-resistant strains (12), Kerr and Panagopoulos (13) described the isolation of new agrocin-producing strains from soil. One new strain was found but was ineffective for biological control.

† Address correspondence to: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139.

In view of the fact that not all strains of *A. tumefaciens* found in South Africa are sensitive to agrocin 84, local isolates of *A. tumefaciens* were screened for the production of new agrocins which might be used to extend the biological control of crown gall. Our approach was different from that of Kerr and Panagopoulos (13), who isolated *Agrobacterium radiobacter* subsp. *tumefaciens* strains from soil. We tested isolates obtained from gall areas on plants for the production of agrocins.

We report here the isolation of a strain of *A. tumefaciens*, D286, which produces an agrocin with a broader host range than that from strain 84. The agrocin was partially purified, and its structure and mode of action were compared with those of agrocin 84. The strain spontaneously lost its pathogenicity, and its potential for biological control is discussed.

MATERIALS AND METHODS

Bacterial strains. Strains of *A. tumefaciens* isolated from crown galls on a variety of plants in South Africa and the other strains used are listed in Table 1. Strain C58 harbors a nopaline type Ti plasmid, strain Ach5 an octopine type, and strains D208 and 396 an agropine type. Strain 84 harbors the agrocin 84-encoding plasmid (pAt84a) and a mobilizing plasmid (pAt84b), and strain 396 harbors pAt396, which encodes agrocin 84. All strains tested carry, in addition, a cryptic plasmid. As *A. radiobacter* 84 is a very slow-growing strain, a pathogenic isolate of *A. tumefaciens* carrying the agrocin 84-producing plasmid pAt396 (strain 396) was used. This strain grows much faster, but a Tn*7*-containing derivative, strain 396::Tn*7*, produced higher levels of agrocin 84. Therefore strain 396::Tn*7* was used as a control. Whether the transposon Tn*7* has any connection with the increased levels of agrocin produced by the strain has not been determined.

TABLE 1. *A. tumefaciens* strains

Strain no.	Natural host	Source or reference	Biotype[a]	Sensitivity to agrocin D286[b]
D208	Begonia	Local strain[c]		S
D209	Chrysanthemum	Local strain		R
D210	Chrysanthemum	Local strain		R
D216	Bauhinia	Local strain		R
D236	Bauhinia	Local strain		R
D259	Yellow jasmine	Local strain		R
D276	Eucalyptus	Local strain		S
D285	Eucalyptus	Local strain		R
D286	Eucalyptus	Local strain	1	R
D287	Eucalyptus	Local strain		R
53	Grapevine	Local strain		R
65	Peach	Local strain		S
71	Peach	Local strain	2	R
72	Unknown	Local strain		S
115	Grapevine	Local strain		R
158	Grapevine	Local strain		R
165	Grapevine	Local strain		R
C58		6	1	S
C58Cl[d]		C58 cured of pTiC58	1	R[e]
Ach5		6		S
Ach5C3[d]		Ach5 cured of pTiAch5		R[e]
84[d]		11	2	R
396		M. van Montagu	1	S
396::Tn7		Transposon Tn7 mutagenesis of 396 (M. van Montagu)	1	S

[a] Given when known.
[b] S, Sensitive; R, resistant.
[c] Local strains were obtained from the Department of Plant Protection, Pretoria, South Africa.
[d] *A. radiobacter* strain.
[e] Faint zone of inhibition.

Media. Cultures were grown in Luria broth (LB) or minimal medium (5), except that sodium glutamate was substituted for glucose.

Agrocin production and sensitivity. Two methods were used to test agrocin production and sensitivity. The method of Kerr and Htay (12) involves growing agrocin-producing strains on minimal medium containing sodium glutamine, killing the cells with chloroform, and overlaying them with the strain to be tested for sensitivity. The method of Mayr-Harting et al. (16) used the supernatants from overnight liquid cultures of agrocin-producing strains inoculated into wells cut into plates seeded with the strain to be tested for sensitivity.

Production of agrocin by strain D286. Samples of logarithmically growing cultures of strain D286 in LB were taken at various intervals. The cells were pelleted, and the supernatants were assayed for agrocin activity by the well-plate method of Mayr-Harting et al. (16). The sensitive indicator strain used was a local isolate, strain D208. The bacteriocin activity (in arbitrary units [AU]) was defined as the reciprocal of the highest dilution giving a detectable zone of inhibition. As a control, the production of agrocin by strain 396::Tn7 was determined, with strain C58 used as the sensitive indicator strain.

To determine the location of the agrocin, the cell pellets, obtained at different times as described above, were washed in 1 M NaCl and the supernatant was assayed for bacteriocin activity. The NaCl-washed cells were then suspended in the same volume of 0.02 M Tris-hydrochloride buffer (pH 8.2) and disrupted by sonication at an amplitude of 8 μm for 2 min in an MSE sonicator. The temperature was not allowed to rise above 10°C during the process. The disrupted cells were then centrifuged at 25,000 × *g* for 15 min, and the supernatant was assayed for agrocin activity.

Partial purification of the agrocin produced by strain D286. Cells were harvested at an optical density at 540 nm (OD_{540}) of 1.36, when agrocin activity was maximal (Fig. 1). Charcoal (Riedel-De-Haen Ag, Seelze, Hanover, Germany) was added to the supernatant to give a concentration of 0.5 g/100 ml. The agrocin was absorbed to the charcoal by stirring at 4°C for 24 hours, and the charcoal was removed from the supernatant by centrifugation at 20,000 × *g* for 15 min. The agrocin was eluted from the charcoal with two changes of 70% ethanol over 24 h at 4°C and concentrated by rotary evaporation of the agrocin-containing ethanol extract at 37°C. The agrocin was separated on a DEAE-Sephadex A25 column, using a stepwise gradient of 0.05 to 0.5 M ammonium phosphate (pH 6.3). As preliminary tests indicated that agrocin D286 might be a nucleotide similar to agrocin 84, absorption at 264 nm was monitored, and the peaks were assayed for agrocin activity. Samples containing partially purified agrocin were concentrated by lyophilization.

Physical characterization of agrocin D286. The temperature stability of the crude and partially purified agrocin preparations was tested at 4, 26, 37, 45, and 60°C. The stability of the crude agrocin at different pHs was determined by mixing equal volumes of the supernatant of the strain D286 culture and 50 mM Tris-hydrochloride; the pH ranged from 2 to 12. The agrocin was incubated at 26°C for 0, 3, or 22 h, and the pH was checked before the culture was assayed. Partially purified agrocin (25 mg/ml = 4 AU) was dissolved in 50 mM Tris-hydrochloride at various pHs and tested as described above. The UV absorption spectrum of the partially purified preparation was compared with that of strain 84 (20).

Effect of agrocin D286 on protein and nucleic acid syntheses. An overnight LB culture of strain D208 was diluted 1:10 and grown in LB at 26°C on an orbital shaker until an OD_{540} of 0.5, as measured on a Spectroplus spectrophotometer, was reached. The culture was divided in two. Agrocin was added to one of the samples, and 0.2 M ammonium phosphate was added to the control. Logarithmic-phase cultures of strain D208 were assayed for protein synthesis in the presence and absence of agrocin D286 by the incorporation of [^{14}C]leucine (2 μCi/ml) into trichloracetic acid (TCA)-precipitable material. Samples (0.1 ml) were removed at intervals, added to 0.1 ml of cold 10% (wt/vol) TCA containing 1 mg of unlabeled leucine per

ml, and kept on ice for 30 min. The samples were filtered on Whatman glass fiber filters (GF/C), and the precipitates were washed with cold 5% (wt/vol) TCA and then with 1% (vol/vol) acetic acid. Radioactivity in the dried filters was counted with a Packard Tricarb scintillation counter.

The effect of agrocin on total nucleic acid synthesis was determined by the incorporation of [^{3}H]adenine (1 μCi/ml) into TCA-precipitable material with the above procedure, except that samples were added to cold 10% (wt/vol) TCA containing 1 mg of unlabeled adenine per ml. DNA synthesis was determined by adding 0.1 ml of 1 M NaOH to 0.1-ml samples, which were then incubated overnight at 37°C to hydrolyze the RNA and proteins. The samples were then neutralized with 7.5 μl of 1% (vol/vol) acetic acid, and 200 μl of cold 10% (wt/vol) TCA containing 1 mg of unlabeled adenine per ml was added to the samples before filtration. RNA synthesis was calculated by the difference between total nucleic acid and DNA counts. As confirmation, DNA synthesis was also determined by the incorporation of [^{3}H]thymine (1 μCi/ml) into TCA-precipitable material. The procedure was the same except that TCA containing 1 mg of thymine per ml was added to the samples. The concentration of agrocin used in the labeling experiments was 10 mg/ml. This amount of agrocin gave 2 AU of agrocin activity.

Plasmid screen. Strains were screened for the presence of plasmids by the method of Kado and Liu (9).

Pathogenicity tests. Strains grown in LB were applied to potato slices, which were incubated on agar plates (0.8% water agar containing 50 U of nystatin per ml, sealed with parafilm to retain moisture) at 26°C and screened for the presence of gall tissue after 8 to 14 days. Virulent and agrocin-producing strains were mixed in different ratios to determine the effect that low numbers of agrocin-producing cells have in controlling tumor formation. Each strain or mixture of strains was applied to six individual potato slices. Heat-killed strain D286 cells were mixed with an equal number of strain C58 or D208 cells to determine whether agrocin or competition for attachment sites was responsible for the inhibition of gall formation.

RESULTS

Production of agrocin by strain D286. Of 18 local isolates of *A. tumefaciens* tested, 6 were found to produce an agrocin which inhibited the growth of at least one other isolate. One strain, D286, was chosen for further study because it inactivated four of the local strains tested, as well as strains 396, 396::Tn*7*, C58, and Ach5. The production of agrocin by strain D286 in LB was compared with the production of agrocin 84 by strain 396::Tn*7* (Fig. 1). All the agrocin activity was found to be extracellular. No agrocin was found in the NaCl solution used to wash the cells, and no further activity was found in disrupted cells. Since agrocin activity disappeared after the peak, different cell fractions of a strain D286 culture were assayed for a possible agrocin-degrading substance. Crude agrocin in LB was mixed 1:1 with whole cells (washed in 1 M NaCl or unwashed), sonicated cells, or supernatants obtained from cells grown to an OD_{540} of between 0.72 and 1.69, encompassing the growth stage during which agrocin degradation occurred. The agrocin and individual fractions were incubated for 16 h at 26°C. Each sample was assayed for agrocin activity. No agrocin-degrading activity was found in any of the fractions tested.

Plasmid-encoded sensitivity to agrocin D286. As preliminarly tests had shown that strains cured of the nopaline Ti plasmid pTiC58 and the octopine Ti plasmid pTiAch5 were resistant to agrocin D286, a series of mutations at various sites on pTiC58 were tested to localize the region encoding sensitivity to the agrocin produced by strain D286. These were deletion mutations derived by the insertion of Tn*1*, Tn*7*, or RP4. Deletion of the plasmid region encoding agrocin sensitivity caused the strain harboring that mutant plasmid to become resistant to the agrocin. Strains harboring plasmid 4005, 4001, 3196, 3178, 3170, or 3101, all of which carry deletions in the region from 64 through 132 to 11 megadaltons (Md), all remained sensitive to agrocin D286. However, when deletions occurred in the region from 11 to 18 Md (plasmids 3190, 3819, and 3116), the strains harboring these plasmids became resistant; indicating that agrocin sensitivity maps in this region (Table 2).

Physical characteristics of agrocin D286. Partially purified and crude agrocin was stable at 4 and 26°C for at least 90 h. Both preparations were inactivated after 22 h of incubation at 37 or 45°C and after 3 h of incubation at 60°C. Both crude and partially purified agrocin D286 was stable at pH 3 to 9 after 22 h of incubation. However, the agrocin was inactivated after 90 h at either pH 3 or 9. The agrocin preparations were resistant to DNase, RNase, and protease. The UV absorption spectrum of partially purified agrocin D286 is shown in Fig. 2.

Mode of action of agrocin D286. DNA, RNA, and protein syntheses in strain D208 cells, as measured by the incorporation of [^{3}H]thymidine, [^{3}H]adenine, and [^{14}C]leucine, respectively, were inhibited after the addition of partially purified agrocin D286 (Fig. 3).

Plasmid screens. Strain D286 was originally isolated as a pathogenic strain from a crown gall on a eucalyptus tree. However, when it was discovered that it produced an agrocin and it was reinoculated into plants, it was found to have lost its pathogenicity. This could have been due either to the complete loss of an inherently unstable Ti plasmid or to the deletion of regions encoding its oncogenic functions. As it was of interest to determine which of these possibilities had occurred and whether agrocin production might be plasmid determined in strain D286,

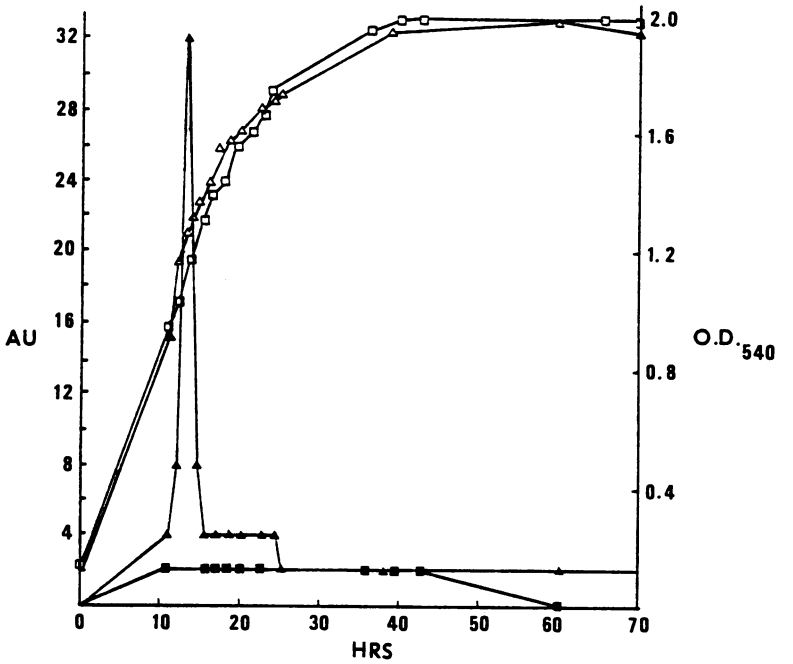

FIG. 1. Comparison of growth curves (open symbols) and the production of agrocin (solid symbols) by strains D286 (△, ▲) and 396::Tn*7* (□, ■). Agrocin activity is expressed in AU (see the text).

plasmid screens of strains D286, 396::Tn*7*, and 84 were compared (Fig. 4).

Strain D286 was found to harbor two plasmids (lane C). One of these, presumably the cryptic plasmid, had the same relative mobility as the 300-Md cryptic plasmid from strains C58, C58C1, and 396::Tn*7* (lanes E, F, and B). The other plasmid was somewhat smaller than the 132-Md Ti plasmid from strains 396::Tn*7* and C58 (lanes B and E), but considerably larger than the agrocin-encoding plasmids from strains 84 and 396::Tn*7* (lanes A and B). The cryptic and the Ti plasmids from the virulent strain D208 (lane D) were both smaller than the corresponding plasmids from strains C58 and 396::Tn*7* (lanes E and B). The chromosomal DNA can be seen as relatively diffuse bands running ahead of

TABLE 2. pTiC58 deletion mutants and their sensitivity to agrocins[a]

Plasmid no.	Deletion generated by:	Approx area deleted (Md)[b]	Sensitivity[c] to agrocin from strain:	
			D286	84
4005	RP4	69–130	S	R
4001	RP4	64–130	S	R
3196	Tn*7*	82–115	S	R
3178	Tn*1*	98.2–102.4	S	S
3170	Tn*1*	125–130	S	S
3101	Tn*1*	131–11	S	S
3190	Tn*7*	99–13.2	R	S
3819	Tn*7*	5–18	R	S
3116	Tn*7*	6–17.4	R	S

[a] Agrocin-producing strains were patched onto minimal medium plates containing sodium glutamate. Plates were incubated for 48 h at 26°C. Individual mutants were seeded as an overlay onto each plate.

[b] Deletion mutants of the nopaline plasmid pTiC58 were isolated by using Tn*1*, Tn*7*, or RP4 (7). The plasmid is 132 Md. The approximate positions of the deletions are given clockwise according to the map. For example, plasmid 3190 has the region from 99 through 132 to 13.2 Md deleted.

[c] S, Sensitive, R; resistant.

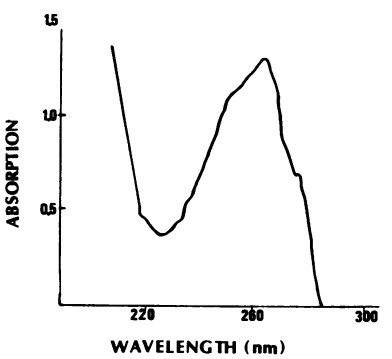

FIG. 2. UV absorption spectrum of partially purified agrocin D286.

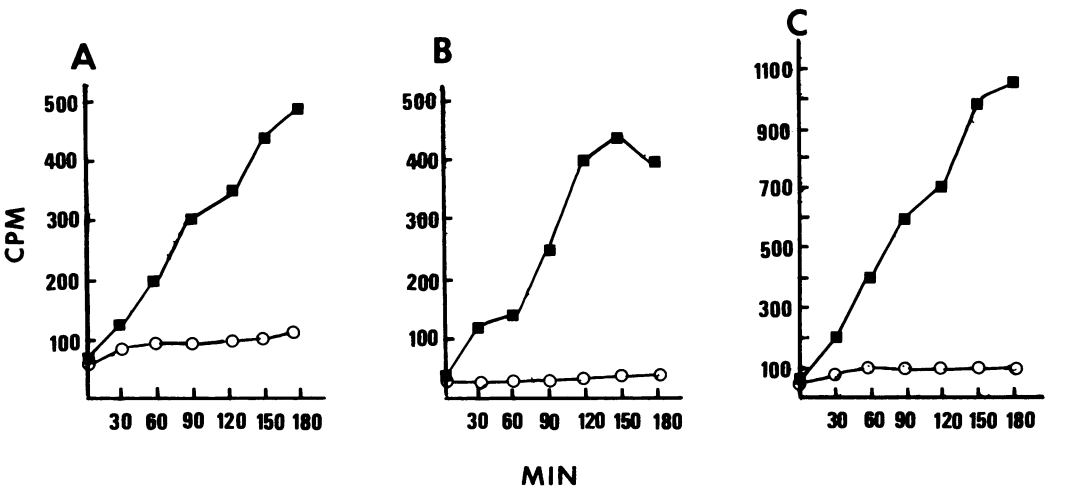

FIG. 3. Effect of agrocin D286 on (A) DNA, (B) RNA, and (C) protein syntheses in a sensitive *A. tumefaciens* strain D208. Incorporation of [^{3}H]thymine, [^{3}H]adenine, and [^{14}C]leucine (panels A, B, and C, respectively) into strain D208 cells (■) and strain D208 cells with agrocin (○) was determined. Samples were taken at intervals, added to 10% TCA, filtered onto glass fiber filters, washed, and counted.

the cryptic and Ti plasmids. The fastest-migrating band in lane B is also chromosomal DNA.

Pathogenicity tests. When stationary-phase cultures of strain D286 were mixed at different ratios with pathogenic strains harboring nopaline, agropine, or octopine plasmids that had been shown to inhibit its growth in vitro, the formation of crown gall tissue on potato slices was inhibited (Table 3). The inhibition was effective with an eightfold excess of strain C58 and a fivefold excess of strain D208. Strain 84, however, was found to inhibit tumor formation by strain C58 only when equal numbers of cells from the two strains were inoculated. When heat-killed strain D286 cells were mixed with equal numbers of strain C58 and D208 cells, gall formation on potato slices was not inhibited.

DISCUSSION

Although a number of local isolates of *A. tumefaciens* were found to produce agrocins, only one, D286, had a broader host range than the agrocin from *A. radiobacter* 84. It was active against *A. tumefaciens* strains harboring nopaline, octopine, or agropine Ti plasmids, whereas agrocin 84 is only active against strains harboring nopaline Ti plasmids (5, 14).

The amount of agrocin produced by strain D286 was markedly higher than the amount of agrocin 84 produced by strain 396::Tn*7* (Fig. 1), which in turn was higher than the amounts produced by strains 396 and 84 (data not shown). However, the activity of agrocin D286 rapidly disappeared after further incubation of the cul-

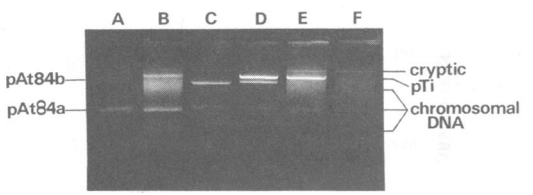

FIG. 4. Agarose gel electrophoresis of the plasmids from strains (A) 84, (B) 396::Tn*7*, (C) D286, (D) D208, (E) C58, and (F) C58C1. Cleared lysates were subjected to electrophoresis through a 0.7% agarose slab gel. The gel was stained with ethidium bromide and photographed on a short-wave UV transilluminator.

TABLE 3. Inhibition of tumor production on potato slices by agrocin-producing strains

Strain tested	Cell ratio (test strain to strain D286)	Tumor formation[a]
C58	2.1:1	–
	4.2:1	–
	8.3:1	–
	10:1	+
D208	1.25:1	–
	2.5:1	–
	5:1	–
	7.5:1	+
Ach5	1:1.5	–
	1.3:1	+
	2.6:1	+
	4:1	+

[a] The assay for tumor formation was qualitative. –, No tumor formation on any of the six potato slices tested; +, tumor formation on all six slices tested.

ture, although the agrocin present in the supernatant fluid after the pelleting of the cells (i.e., the crude extract) was extremely stable. No agrocin-degrading function could be found associated with the cells.

Sensitivity to agrocin D286 was found to map in the region from 11 to 18 Md on the nopaline Ti plasmid pTiC58. The exclusion of phage AP1 also maps in this region of the plasmid, but whether there is any relationship between these functions is not yet known. Although sensitivity to agrocin D286 is also encoded on the octopine Ti plasmid pTiAch5, its position has not yet been mapped. However, it is of interest to note that the region of pTiC58 in which sensitivity to strain D286 mapped fell within one of the homologous regions between pTiC58 and the octopine plasmid pTiB6S3.

Analysis of the sensitivity to agrocin D286 of strains harboring mutant Ti plasmids or of strains cured of the Ti plasmid was initially hampered by the occasional appearance of extremely faint zones of inhibition around some strains when tested by the method of Kerr and Htay (12). This was particularly noticeable when sodium glutamate was substituted for glucose in the medium. Glucose appeared to inhibit the production of both agrocins D286 and 84 on plates. From the results (Table 1) it appears that although sensitivity to agrocin D286 is indeed encoded by the Ti plasmid, another plasmid-independent uptake system may exist which is considerably less efficient and only rarely noticeable.

The physical characteristics of the partially purified agrocin D286, in particular its UV absorption spectrum (Fig. 2), were consistent with its being a nucleotide. Agrocin 84 is also a nucleotide, a disubstituted fraudulent nucleotide with a 9-(3'-deoxy-α-D-threo-pentafuranosyl)adenine core (20). The mode of action of the two agrocins was also similar. Agrocin D286 inhibited DNA, RNA, and protein syntheses (Fig. 3), and Das et al. (2) showed that agrocin 84 inhibits DNA synthesis at low concentrations, whereas RNA and protein syntheses are affected at higher concentrations.

The discovery that strain D286 had lost its pathogenicity was surprising, and plasmid analysis revealed the presence in it of a 300-Md cryptic plasmid and a plasmid with a somewhat lower molecular weight than the Ti plasmid of strains C58 and 396::Tn7 (Fig. 4). This smaller plasmid might be the Ti plasmid that had the regions encoding its oncogenic functions deleted, or it might be a plasmid coding for agrocin D286 production. However, lack of pathogenicity is essential before strain D286 can be tested for the biological control of crown gall, and thus this spontaneous loss is of practical value. The advantages of strain D286 over strain 84 are its activity against strains harboring nopaline, octopine, and agropine Ti plasmids and its faster growth rate. (The doubling time at 26°C of strain D286 in LB supplemented with 0.5% Casamino Acids was 6.5 h, whereas that of strain 84 was 12 h. As poor growth of agrocin-producing strains at the wound site might be a reason for their inutility as biological controls (3), a strain with a faster growth rate which produces high levels of agrocin might well be an advantage. Strain D286 prevented the development of crown gall tissue on potato slices by strains sensitive to its agrocin. It was able to do this even in the presence of an eightfold excess of strain C58 and a fivefold excess of strain D208 cells. Strain 84 prevented tumor formation under the same conditions only when an equal number of its cells were added to cells of strain C58. That tumor growth was inhibited by the production of agrocin D286 and not by the competition of strain D286 cells for attachment sites was shown when heat-killed strain D286 cells failed to prevent gall formation.

In view of the potential of strain D286, it is at present under investigation as an agent for the biological control of crown gall.

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