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Utilization of Octopine and Nopaline by *Agrobacterium*

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Tests for utilization of D-octopine and nopaline in defined media containing a carbon and nitrogen source were made on 60 strains of *Agrobacterium* representing four species and on a representative of each of five species of *Rhizobium*. Among 46 virulent strains of *Agrobacterium*, only two strains were found which utilized neither compound, while three strains were found which could utilize both. Of the remaining virulent strains, 27 utilized octopine and 14 utilized nopaline. Each of six strains of *A. rhizogenes* tested utilized only octopine but at a slower rate relative to growth than most *A. tumefaciens*. All eight of the *A. radiobacter* strains failed to utilize either compound, as did four of six nonvirulent strains of *A. tumefaciens*. The rhizobia did not utilize octopine or, with the possible exception of *R. japonicum*, nopaline. Virulence in the genus *Agrobacterium* is concluded to be highly correlated with the ability to utilize one or both of these compounds.

The demonstration by Goldmann et al. (1) that bacteria-free crown-gall tumors induced by *Agrobacterium tumefaciens* strain T-37 form nopaline whereas bacteria-free tumors induced by strain B6 form octopine has opened an important new avenue of investigation in the crown-gall field. Petit et al. (9) subsequently reported that tumors induced by 43 different strains of *A. tumefaciens*, with but four exceptions, produced detectable amounts of either octopine or nopaline. This unique difference, which depends on the strain of bacterium used to incite the tumor, was found to correlate with the ability of the bacteria to degrade octopine or nopaline in six test strains (9). Thus, bacteria which induce octopine-forming tumors degrade octopine but not nopaline, and vice versa. More recently Petit and Tourneur (10) have reported that loss of virulence in strain B6 is accompanied by loss of the ability to degrade octopine.

The importance of these compounds in the crown-gall syndrome was further emphasized by the discovery that they promote tumor growth in vivo (4, 5). In general, the tumors show growth responses to only the compound which they produce (5; and unpublished observations). Thus, octopine or nopaline formation by the tumor tissue may be inferred to contribute to the abnormal growth which characterizes the tumor. To provide a basis for extending these physiological studies and to assess the overall importance of octopine and nopaline utilization to the virulence and characterization of the genus *Agrobacterium*, we have compared the ability of representative strains of agrobacteria to degrade octopine and nopaline. Most virulent strains of agrobacteria are found to utilize either octopine or nopaline, whereas most avirulent strains, including all strains of *A. radiobacter* tested, fail to degrade either of these compounds.

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MATERIALS AND METHODS

Bacterial strains. Each of the strains of *A. radiobacter*, *A. rhizogenes*, and *A. rubi* described previously (3) was utilized in these tests, along with the majority of *A. tumefaciens* described there. In addition, tests were run on *A. tumefaciens* strains EU6 and 181, kindly supplied by G. Morel and J. Tourneur, and strains AT-1 (Dahlia Rabitz 1a), AT-3 (Pappel 3), and AT-4 (Dahlia Rabitz 1b) were obtained from the Institute of Bacteriology, Biol. Bundesanstalt, Berlin-Dahlem. *Rhizobium meliloti* strain 102F28, *R. japonicum* strain 61A72, *R. trifolium* strain 162K4, *R. phaseoli* strain K-17, and *R. leguminosarum* strain C-56 were graciously supplied by H. J. Evans.

Culture media and conditions. All *Agrobacterium* cultures were maintained on the nutrient broth-yeast extract-sucrose medium as described elsewhere (3), and *Rhizobium* cultures were maintained on yeast

extract-mannitol medium (6). To prepare inocula for utilization studies, 50 ml of liquid medium S of Monod (8) was inoculated from a slant and incubated for 48 h at 27 C on a reciprocal shaker. This medium was supplemented with (per liter): 10 mg of biotin, 10 mg of nicotinic acid, 10 mg of calcium pantothenate, and 1 g of glutamate for auxotrophic strains of *Agrobacterium* (3). *Rhizobium* strains were grown on the defined medium of Rigaud (11) under the above conditions. In Monod's medium, glucose was autoclaved separately and added to sterile salt solutions. Octopine and nopaline were sterilized by filtration through membrane filters (Millipore) of 0.22-μm pore diameter.

Based on the procedures of Petit et al. (9), 0.4 ml of the cultures grown on medium S was added to 3.0 ml of medium S with supplements as appropriate and containing 50 μg of either octopine or nopaline per ml. In experiments with natural nopaline, one-fifth this amount was used. In some experiments aeration was supplied by bubbler tubes, and the cultures were grown in tubes (16 by 150 mm); in others they were shaken as above in 50-ml Erlenmeyer flasks with Bellco stainless-steel covers. Both procedures yielded similar results.

Determination of octopine and nopaline. After 24 h of growth in octopine- or nopaline-containing media or at times indicated in the text, the cultures were centrifuged at 10,000 $\times$ *g* for 15 min to sediment the bacteria, and about 2 ml of the supernatant fluid was carefully removed for subsequent analysis. The presence of octopine or nopaline in the medium was determined in initial experiments by the α-naphthol-diacetyl procedure for detection of guanidino compounds as described by Ménagé et al. (7). A procedure adapted from Rosenberg et al. (12) was utilized in later experiments since it proved more sensitive. To a 1-ml sample of culture supernatant fluid, 1 ml of 0.9 N NaOH was added with mixing, followed by 1 ml of α-naphthol-diacetyl reagent (3% α-naphthol plus 0.015% diacetyl in 60% *n*-propanol) with mixing. The reactants were allowed to stand at room temperature for 30 min for optimal color development, and their absorbancy was determined in a Gilford spectrophotometer at 530 nm. As the color does not fully stabilize but continues to increase slowly in both blank and octopine- or nopaline-containing samples, the addition of reagents to samples was staggered to accommodate the time required for absorbancy determinations when many samples were to be assayed. The absorbancy at 530 nm of the blank (culture medium plus reagents) typically fell between 0.150 and 0.250, and that of the medium plus octopine or nopaline fell between 1.050 and 1.200.

Culture growth was measured with a Bausch and Lomb Spectronic 20 at 660 nm. D-Octopine and α-naphthol were obtained from Sigma Chemical Co., Inc. Nopaline was synthesized by the procedure of Thoai and Robin (13) and purified by chromatography on SE-Sephadex C-25. With few exceptions, a maximum of only 25 to 35% of the synthetic nopaline was utilized in most experiments. The nopaline results were confirmed by utilizing a crystalline sample of natural nopaline generously provided by G. Morel (2) which, at one-fifth the concentration of the above, was fully utilized by positive strains in 24 h.

RESULTS

Initial experiments indicated some strains of *Agrobacterium* consistently utilized all of the octopine in the medium after 24 h of growth, and others utilized none of the octopine. A few strains, however, and in particular all of the *A. rhizogenes* strains, showed only partial utilization within this period. Time courses (Table 1) contrasting bacterial growth with octopine utilization were run to determine whether these differences in utilization represented maximal values or were simply due to different rates of utilization. Strains such as P2 or 398 of *A. tumefaciens* and ATCC 15834 and ATCC 11325 of *A. rhizogenes* completely utilized the octopine after longer incubation periods. Strains which showed no utilization at 24 h, however, did not show significant octopine utilization after 32 h of incubation and, though not shown in the table, after 48 h. Utilization of octopine by the different *Agrobacterium* strains, while characteristic of the strain, was not obviously correlated with any particular stage in the growth of the different cultures.

Because low rates of octopine utilization might be attributable to mixed cultures containing utilizing and non-utilizing strains, single-colony isolates were obtained from several strains which slowly utilized octopine, and the tests were repeated. While the amount of octopine utilization by various isolates varied somewhat in different tests, all showed definite utilization and at a slow rate as compared with strains such as B6.

Table 2 summarizes the results of tests on 60 strains of *Agrobacterium* and 5 strains of *Rhizobium* for octopine and nopaline utilization. Among the tumorigenic *A. tumefaciens*, all strains obviously derived from another (e.g., B6-806, ATCC 11158, ATCC 11156, B6-6, each derived from B6) were deleted from the table, and only the results obtained with the parent strain were included. Each of the five strains derived from B6, however, gave identical results to those obtained with B6. In the case of octopine utilization, 46 of the 60 *Agrobacterium* strains were tested in at least three separate growth experiments using at least two separately prepared starter cultures. Of the 47 *Agrobacterium* strains tested for nopaline utilization, 33 were tested two or more times with two separate inocula. The rhizobia were tested twice for octopine utilization and once for

TABLE 1. *Time course of growth and octopine utilization by certain strains of Agrobacterium*

Organisms	16 h[a]		20 h		24 h		28 h		32 h	
	Abs[b] at 660 nm	Util[c] (%)	Abs at 660 nm	Util (%)	Abs at 660 nm	Util (%)	Abs at 660 nm	Util (%)	Abs at 660 nm	Util (%)
A. tumefaciens										
B6	0.310	100	0.340	100	0.355	100	0.370	100	0.315	100
ATCC 15955	0.230	100	0.300	100	0.305	100	0.300	100	0.310	100
P2	0.235	10	0.325	37	0.350	68	0.350	87	0.315	100
398			0.630	23	0.640	54	0.635	92	0.640	100
T-37	0.090	0	0.160	0	0.195	0	0.250	0	0.255	0
H-100	0.255	0	0.335	0	0.360	0	0.355	0	0.325	10
181			0.325	0	0.440	0	0.490	0	0.540	0
IIBV7			0.420	0	0.470	0	0.485	0	0.545	0
A. rhizogenes										
ATCC 15834	0.180	0	0.315	16	0.345	52	0.350	100	0.305	100
ATCC 11325	0.150	7	0.265	11	0.280	43	0.305	73	0.280	100

[a] Hours of incubation.
[b] Abs, Absorption.
[c] Util, Utilization.

TABLE 2. *Summary of octopine and nopaline utilization by Agrobacterium and Rhizobium sp.*

Type organisms	No. of strains tested/ no. showing utilization			
	Octopine	Nopaline	Both	Neither
Virulent *A. tumefaciens*	38/25	25/15	25/3	38/2
Avirulent *A. tumefaciens*	6/2	6/0	6/0	6/4
Virulent *A. rubi*	2/0	2/2	2/0	2/0
Virulent *A. rhizogenes*	6/6	6/0	6/0	6/0
Avirulent *A. radiobacter*	8/0	8/0	8/0	8/8
Rhizobium sp.[a]	5/0	5/1	5/0	5/4

[a] Received as effective strains but not tested for this ability.

nopaline utilization. A clear difference existed in the amount of these compounds recovered in the growth medium between strains scored as utilizing a particular compound and strains scored as showing no utilization. Only four strains scored as showing utilization (other than the six strains of *A. rhizogenes*) failed to utilize 70% or more of the octopine or natural nopaline in the 24-h incubation period. Less than 10% of the octopine or nopaline was removed from the supernatant fluid in most tests on strains scored as failing to use these compounds. The few cases of questionable utilization were easily resolved by testing for utilization after longer periods of incubation.

Nearly two-thirds of the virulent *Agrobacterium* strains showed octopine utilization. Due to limitations of the nopaline supply, only representative strains which showed full octopine utilization at 24 h were tested for nopaline utilization (all failed to show nopaline utilization) along with all strains which failed to use octopine or which showed only partial utilization. Consequently, a higher proportion of the strains tested for nopaline utilization were positive than could be expected in randomly selected strains. Only 2 of the 46 virulent strains failed to utilize either octopine or nopaline, while three strains were found which utilized both. The two strains of *A. rubi* utilized nopaline and not octopine, whereas the six strains of *A. rhizogenes* all showed the reverse of this utilization pattern. On a percentage basis only 4% of the virulent agrobacteria could degrade neither compound and only 7% appeared to degrade both.

Each of the eight strains of *A. radiobacter* and four of the six nontumorigenic strains of *A. tumefaciens* failed to degrade either compound. Thus, avirulence shows a high degree of correlation with the inability to degrade these compounds and virulence is highly correlated with the ability to degrade them. None of the rhizobia showed octopine utilization although good growth was obtained and in one experiment the incubation was continued to 48 h. Only *R. japonicum* of the five rhizobia tested showed partial utilization of nopaline in a single test.

Three strains were found which could utilize both octopine and nopaline. Single-colony isolates of strain Ag6 all utilized both octopine and

nopaline as does the parent strain, consuming 34 to 78% of the octopine and 85 to 100% of the nopaline in the 24-h incubation period. *A. tumefaciens* strains TT133 and P2 also degrade both compounds, and single-colony isolates of strain TT133 all utilize octopine. The ability to utilize both compounds, therefore, while uncommon, need not be due to trivial cause such as a mixed culture. These three strains have two additional characters in common which are somewhat atypical, each is auxotrophic and each fails to produce 3-ketoglycosides (3).

The tumorigenic strains AT-1 and AT-4 were the only virulent agrobacteria which appeared unable to degrade either compound. Aside from their similar origin and inability to grow on a minimal medium, these two strains are unusual because they fail to induce tumors on pinto bean leaves, yet readily induce tumors on carrot root discs. All strains of agrobacteria examined to date which are tumorigenic on pinto bean leaves degrade either octopine, nopaline or both.

As a group, the six *A. rhizogenes* strains were unique in that they all utilized octopine, and most tests showed only 30 to 60% octopine utilization in the standard 24-h incubation period. Auxotrophic strains of *A. tumefaciens* grown on the same supplemented medium as that used for the *A. rhizogenes*, however, typically degraded 80 to 100% of the octopine. As illustrated in Table 1, these *A. rhizogenes* strains all showed complete utilization of the octopine with longer incubation periods.

The two nonvirulent strains of *A. tumefaciens* which showed octopine utilization were strains 5GlyFe and Ag19. The former was derived by glycine attenuation (14) from virulent strain A6, an octopine-utilizing strain, of *A. tumefaciens*. Loss of the capacity of strain 5GlyFe to initiate tumors, therefore, apparently occurred without loss of octopine-utilizing ability. Strain Ag19 was isolated from a grape vine gall and, except for its failure to initiate tumors, shows all the characteristics expected of typical strains of *A. tumefaciens* (3).

Avirulent strain IIBNV6 presents an interesting case in that it was derived by A. Braun from the same parent culture as strain IIBV7. Petit et al. (9) report that the latter strain utilizes nopaline, which we have confirmed, but they were unable to detect nopaline utilization by strain IIBNV6. In our tests, strain IIBNV6 showed partial utilization of synthetic nopaline but failed to utilize natural nopaline. This difference may result from utilization of the unnatural nopaline isomer by strain IIBNV6 (the synthetic product is probably a mixture of DL-nopaline), and failure to utilize the natural isomer although the stereochemistry of the latter is not resolved.

DISCUSSION

The ability to utilize either octopine or nopaline provides the best current diagnostic test short of plant inoculations for distinguishing virulent from avirulent strains of *Agrobacterium* (3) despite the few exceptional strains found. The fact that all strains of *A. radiobacter* and four of six strains of non-tumorigenic *A. tumefaciens* failed to show utilization is clear evidence that, in nature, lack of virulence is commonly associated with the inability to degrade either of these compounds. Thus, the finding of Petit and Tourneur (10) that loss of virulence by strain B6 in culture is accompanied by loss of the ability to degrade octopine may account for the remarkable similarities between *A. tumefaciens* and *A. radiobacter* since the latter species is both avirulent and apparently unable to utilize either of these unusual arginine derivatives. Our results show, however, that all possible types based on octopine and nopaline utilization are to be found among the tumorigenic agrobacteria, octopine utilizers, nopaline utilizers, utilizers of both, and utilizers of neither. The latter two types though are clearly a minority of the strains tested.

Petit et al. (9), in examining the tumors induced by 43 different strains of *A. tumefaciens*, found that essentially half of the tumors produced octopine, and with few exceptions the remainder formed nopaline. About 65% of the tumorigenic cultures in our tests were able to utilize octopine, and most of those remaining could utilize nopaline. In nature the distribution of these two major types of tumorigenic agrobacteria, therefore, would appear to be widespread and to approach equal frequency. Host preferences, however, such as those commonly ascribed to *A. rubi* and *A. rhizogenes* may preferentially select for one of these pathogen types.

Two strains of *A. tumefaciens*, EU6 and 181, were reported by Petit et al. (9; G. Morel, personal communication) to produce tumors which contained no detectable octopine or nopaline. In the present experiments, both of these strains were found to utilize nopaline but not octopine. The failure of strain 181 and EU6 tumors to show increased growth on addition of octopine or nopaline (Lippincott and Lippincott, unpublished data), however, correlates with the results of Petit et al. (9). It appears, therefore, that, while these strains utilize nopa-

line, they fail to induce nopaline-forming tumors. Consequently, the ability to utilize octopine or nopaline by a particular bacterial strain is not always indicative of production of this compound by tumors induced by the same strain. To date, however, these two strains appear to be the only exceptions.

Medium S in which these tests were run contains glucose and an inorganic nitrogen source providing organisms cultured in it the choice of utilizing the carbon and nitrogen of these sources for growth versus that of additional compounds supplied in the medium (8). The fact that some strains utilized octopine throughout the major part of their growth cycle while others, such as the *A. rhizogenes* strains, only utilized octopine in late stages of growth may indicate a basic difference in the control of the pathway regulating octopine utilization. This pathway would appear to be nonconstitutive in those strains which only utilize octopine in late stages of growth and to be induced only after other substrates are depeleted. This difference may contribute to the host reactions which distinguish *A. tumefaciens* and *A. rhizogenes* infections.

Of the strains which gave anomalous results, (i.e., AT-1, AT-4, Ag6, TT133, P2, EU6, and 181) only one, strain 181, was prototrophic. Deviations from the norm in octopine and nopaline utilization, therefore, may be expected most frequently in auxotrophic strains.

Since D-lysopine is also formed by octopine-producing tumors but not by nopaline tumors (9), several octopine- and nopaline-utilizing strains were also tested for lysopine utilization. In general, octopine strains degraded most of the lysopine within 24 h, while most nopaline strains utilized only 30 to 50% of the lysopine. Because the ninhydrin test used to detect lysopine utilization was influenced by the NH_3 content of the medium as well as by other ninhydrin-positive metabolites, further studies of its utilization were discouraged. Carnosine utilization was also studied since this dipeptide promotes crown-gall tumor growth similar to octopine, lysopine, and nopaline. Most of the strains tested utilized from 30 to 70% of the carnosine, but utilization was not correlated with the ability of different strains to induce a carnosine-like growth factor or with octopine or nopaline utilization (5).

Because the rhizobia tested failed to utilize octopine, and with one exception nopaline, the results suggest these compounds probably do not occur or have physiological significance in *Rhizobium*-induced nodules. Direct tests for these compounds in nodules should be made before this question is closed, however. These results do provide an additional criterion distinguishing the agrobacteria from the rhizobia.

While many strong generalizations can be drawn from these studies and those of Petit et al. (9) and Petit and Tourneur (10), few appear to be without exception. As evidence of these exceptions, certain strains can utilize both octopine and nopaline, certain strains which induce tumors are unable to utilize either, certain strains which degrade nopaline initiate tumors which are unable to form nopaline, certain non-tumorigenic strains can utilize one of these compounds, and not all exceptional strains are auxotrophic. Without exception, however, all *A. rhizogenes* strains utilized octopine, but not nopaline, and all *A. radiobacter* strains failed to utilize either. Since virulent *A. tumefaciens* exist which cannot utilize octopine, similar exceptions may be expected in *A. rhizogenes* although it appears less probable that *A. rhizogenes* strains will be found which degrade nopaline. There is currently no effective way to distingush avirulent *A. tumefaciens* strains which may degrade one of these compounds from an *A. radiobacter* even if all "true" radiobacters actually lack this characteristic. Consequently, strains of *A. radiobacter* may be anticipated which will degrade octopine or nopaline. As a temporary convention, it may prove useful to consider all *Agrobacterium*-like strains which are non-tumorigenic as *A. radiobacter* if they fail to degrade either octopine or nopaline and those avirulent strains which utilize one of these compounds as *A. tumefaciens*.

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