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Isolation of Different *Agrobacterium* Biovars from a Natural Oak Savanna and Tallgrass Prairie†

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Populations of agrobacteria in excess of 10^5 CFU/g were recovered from 12 soil and root samples obtained from the Allison Savanna, Minn., a natural oak savanna and tallgrass prairie which has never been disturbed agriculturally. Of 126 strains picked randomly from selective media, 54 were identified as *Agrobacterium* spp. Biovar 2 strains predominated (35 of 54), but these strains were distributed into three phenotypically distinct subgroups. Of the remaining *Agrobacterium* strains, four were biovar 1-2, one was biovar 1, and none were biovar 3. The last 14 *Agrobacterium* strains formed a homogeneous group which differed biochemically from the hitherto reported biovars. Opine utilization (coded for by genes on the tumor-inducing plasmid in pathogenic *Agrobacterium* spp.) by these agrobacteria was limited to two biovar 2 strains. In contrast, 10 nonfluorescent gram-negative strains utilized either nopaline or octopine as the sole carbon and nitrogen source. There may be a need to reexamine the source and role of opines in the terrestrial environment because (i) all of these opine utilizers were isolated from an environment free of crown gall, the only known terrestrial source of opines, and (ii) 83% of the opine utilizers were not *Agrobacterium* spp.

The ecology of *Agrobacterium* spp. in nonagricultural environments has received little attention, partly because the research by plant pathologists has focused primarily on crown gall, a serious neoplastic disease in plant nurseries caused by pathogenic agrobacteria (28). Such research has shown that *Agrobacterium* spp. are able to successfully invade cultivated soils and cause epidemics (8, 33). Yet in these cultivated soils, the *Agrobacterium* population is predominantly nonpathogenic (15, 33). Although nonpathogenic strains could potentially become pathogens upon acquisition of the conjugative Ti plasmid (pTi) (39), which is responsible for tumor induction (40), most plant pathologists do not consider the nonpathogenic population to be a threat to the development of crown gall epidemics. This perception may be the reason why the biological activity and survival of this organism in nonagricultural soils has not been investigated.

The present study was designed to determine whether *Agrobacterium* strains inhabit soils of a natural environment and, if so, whether their phenotypic characteristics would associate them with the presently recognized biovars (19). For this purpose, soil and root samples were obtained from the Allison Savanna, Minn., a natural oak savanna and tallgrass prairie which has never been disturbed agriculturally. This preserve has been maintained as it was in presettlement days, when such plant communities covered much of the central North American continent.

This paper reports the range of *Agrobacterium* occurrence in the Allison Savanna, the predominance of biovar 2 strains in root and soil samples obtained from this savanna, a hitherto undescribed physiological group of agrobacteria, and the predominance among opine utilizers of bacteria that are not *Agrobacterium* spp.

MATERIALS AND METHODS

Organisms and cultivation. Bacteria were isolated from both roots and soil surrounding plants in the families Fagaceae (*Quercus* sp.), Rosaceae (*Rubus flagellaris*), Fabaceae (*Lathyrus venosus*), Asteraceae, and Poaceae and in an unidentified moss plant (class Muscopsida). All plants sampled were located several hundred meters apart in the Allison Savanna, a preserve of The Nature Conservancy, located in the Anoka Sand Plain of Anoka County, Minn. Individual plants with about 250 g of sandy-loam soil surrounding the root system were placed in plastic bags for transport to the laboratory. Roots were removed and washed free of adhering soil, and 1 g of root tissue was ground with a mortar and pestle for 2 min in 10 ml of sterile distilled water. For the soil samples, 1 g of soil was suspended in 10 ml of sterile distilled water and vortexed for 1 min. The soil suspension was passed through a screen with sieve openings of 0.125 mm to remove plant residues. The six root and six soil suspensions were allowed to settle at room temperature for 5 min before 10-fold serial dilutions of each suspension were made; 0.1 ml of selected dilutions was spread onto 1A, 2E, and 3DG semiselective media for isolation of *Agrobacterium* spp. (6). The inoculated plates were incubated for 1 week at 25°C before population counts were made. A total of 30 colonies (10 from each medium), representing different colony types on the semiselective media, were selected at random from each sample and purified by two successive streakings on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) calcium carbonate. Bacterial cultures were stored at 4°C on PDA-calcium carbonate slants and in sterile distilled water.

Characterization of strains. Strains were examined for their Gram reaction (32) and production of fluorescent pigment on King medium B (21). To determine the biovar affiliation (14, 17, 35) of each putative *Agrobacterium* strain, we performed the following tests: oxidation of lactose to 3-ketolactose (3); oxidase reaction (22); growth and pigmentation in ferric ammonium citrate (12); utilization of citrate (34), L-tyrosine (20), mucic acid, and L-(+)-tartaric acid (37); alkali production from malonate (23); and acid production from erythritol and melezitose (17).

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TABLE 1. Population levels of *Agrobacterium* strains isolated from samples of plant roots and surrounding soil obtained from the Allison Savanna

Colony type[a]	Population levels (10^5 CFU/g) of *Agrobacterium* strains from:											
	Moss		Poaceae		Asteraceae		Fabaceae		Fagaceae		Rosaceae	
	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots
1	–[b]	–	–	–	–	1	–	–	–	–	–	–
2	0.2	0.5	30	80	1	6	100	200	10	2	–	1
1-2	–	–	2	–	–	–	–	–	–	–	40	20
4	1	2	–	90	–	–	–	–	5	–	–	–

[a] Four major distinct colony types were observed on the selective media used for isolation of *Agrobacterium* strains. All four types were pulvinate, smooth, glossy, and circular with an entire margin. The type 1 colony was 4 mm in diameter and gray with an orange center on medium 1A. Type 2 colonies were 2 mm in diameter, and consisted of three subgroups: type 2′, translucide gray on medium 1A; type 2″, translucide green on medium 2E; type 2‴, pink on medium 3DG (all of the type 2 colonies were characterized as physiological group 2). The type 1-2 colony was 3 mm in diameter and pale green on medium 2E. The type 4 colony was about 1 mm in diameter and gray on medium 1A. These colony types correspond to physiological groups shown in Table 2.

[b] –, Not detected at a 10^{-2} dilution.

Utilization of opine as the sole carbon and nitrogen source was determined initially from growth on basal medium solidified with 0.4% (wt/vol) Gelrite (Kelco Division of Merck & Co., Inc., San Diego, Calif.) containing 5 mM octopine (Sigma Chemical Co., St. Louis, Mo.) or nopaline (Sigma) and confirmed subsequently by growth in liquid basal medium containing the same opine concentration. Gelrite was used instead of agar as the gelling agent because the use of agar can result in false-positive data (M. L. Canfield and L. W. Moore, unpublished results). The basal medium consisted of the following: K_2HPO_4, 41 mM; KH_2PO_4, 21 mM; $MgSO_4 \cdot 7H_2O$, 0.8 mM; $CaCl_2 \cdot 2H_2O$, 75 μM; $FeSO_4 \cdot 7H_2O$, 18 μM; and $MnCl_2 \cdot 4H_2O$, 10 μM; the pH was adjusted to 7.2 (Canfield and Moore, unpublished results).

Because some of the putative *Agrobacterium* strains recovered from selective media might be *Rhizobium* strains we inoculated these strains into three species of legumes to test for nodulation. Seeds of alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris* 'Yellow Wax'), and pea (*Pisum sativum* 'Sugar Snap') were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite for 10 min, germinated for 2 days at 25°C, and transferred aseptically to sterile test tubes (30 by 3 cm) containing 20 ml of a mineral salts agar medium (9). The seedlings were inoculated with 0.2 ml of bacterial suspension (10^8 CFU/ml) and cultured in a growth chamber for 6 weeks before the plants were scored for nodulation. *Rhizobium leguminosarum* biovar *phaseoli* 127K12b, *R. leguminosarum* biovar *trifolii* 162S7a, and *R. meliloti* YA-15 were used as positive controls, and sterile distilled water was used as a negative control.

To confirm the identity of the putative *Agrobacterium* strains, purified ribosomes extracted from seven representative strains were reacted in gel immunodiffusion tests with five antisera made against 50S ribosomal subunits of *Agrobacterium* strains B6, C58, M63/79, U11, and CG64 (5).

Pathogenicity tests were performed on stems of 4-week-old seedlings of tomato (*Lycopersicon esculentum* 'Bonny Best') (1), and tumor formation was recorded 8 weeks after inoculation.

Sensitivity to agrocin 84, an antibiotic produced by the biocontrol agent *Agrobacterium* strain K84, was tested by the method of Stonier as modified by Cooksey and Moore (7). Because the genes for agrocin sensitivity are located on the nopaline pTi, this test is helpful as a putative test for pathogenicity (16).

Agrobacterium strains B6 (biovar 1, octopine utilizer) (R. Baker, Colorado State University), C58 (biovar 1, nopaline utilizer) (R. Dickey, Cornell University), K84 (biovar 2) (A. Kerr, Waite Institute, South Australia) and CG64 (biovar 3) (T. Burr, New York State Agriculture Experiment Station at Geneva) were included as reference strains.

RESULTS

Isolation of agrobacteria from both soil and root samples. *Agrobacterium* strains were recovered from all 12 soil and root samples (Table 1). The population of *Agrobacterium* strains in soil ranged from 10^5 to 10^7 CFU/g and was unexpectedly similar to the population on the roots.

Identification of strains. From the three different selective media, 126 bacterial colonies representing all different colony types were selected for further characterization. Fifty-four *Agrobacterium* strains were identified, but none was tumorigenic on tomato seedling nor sensitive to agrocin 84.

Thirty-five strains were biovar 2 agrobacteria, one was biovar 1 and four shared characteristics of biovar 1-2 of Spiers (35). The remaining 14 strains formed a fourth physiological group (Table 2). None of the 12 samples yielded biovar 3 strains.

Although the biovar 2 population was predominant in this study, only 8 of 35 biovar 2 strains were isolated on the biovar 2 semiselective medium 2E. Seventeen of these strains which were unable to grow on medium 2E did not grow on erythritol; they formed a biovar 2 subgroup represented by strain R3/84 (Table 2). Another biovar 2 subgroup, represented by strain S2/84, included 15 L-tyrosine-negative strains. A third biovar 2 subgroup consisted of three strains that shared biovar 1 characteristics; they grew on medium 1A and on ferric ammonium citrate. Biovar 1-2 strains differed from the reference biovar 3 strain by their ability to grow on medium 1A and to utilize erythritol; three of these strains did not grow on biovar 3 medium 3DG.

Ribosomal serology and nodulation assay. Ribosomes from representative strains of each phenotypic group listed in Table 2 reacted positively in immunodiffusion tests with *Agrobacterium* ribosomal antisera. These strains produced a precipitin band which formed a reaction of complete fusion (identity) with the precipitin band of the homolog (i.e., the strain used to develop the antiserum) (Fig. 1). Results of this serological test and the lack of nodulation of the three legume species confirmed that these strains were agrobacteria.

Utilization of opines. Of 126 strains tested, 30 (6 of which were agrobacteria) grew on solid media containing either nopaline or octopine as the sole carbon and nitrogen source (Table 3). However, only 12 (two of which were *Agrobacterium* biovar 2) of the 30 strains grew when they

TABLE 2. Characteristics and distribution of the 54 *Agrobacterium* strains recovered from soil or plant roots from the Allison Savanna

Diagnostic test	Reaction to tests of strains in physiological group[a]:									
	1		2				3	1-2		4
	B6[b]	R6/84[c] (1)[d]	K84[b]	S2/84 (15)	R1/84 (3)	R3/84 (17)	CG64[b]	R5/84 (3)	S5/84 (1)	S4/84 (14)
3-Ketolactose	+	+	−	−	−	−	−	−	−	−
Oxidase	+	+	−	−	−	−	+	+	+	−
Ferric ammonium citrate	+	+	−	−	+	−	−	−	−	−
Citrate	−	+	+	+	+	+	+	+	+	+
L-Tyrosine	−	−	+	−	+	+	−	−	+	−
Mucic acid	−	−	+	+	+	+	−	−	−	−
L-(+)-Tartaric acid	−	−	+	+	+	+	+	+	+	−
Malonate	−	−	+	+	+	+	−	−	−	−
Erythritol	−	−	+	+	+	−	−	+	+	+
Melezitose	+	+	−	−	−	−	−	−	−	−
Growth on medium 1A	+	+	−	−	+	+	−	+	+	+[e]
Growth on medium 2E	−	−	+	+	−	−	−	+	−	+
Growth on medium 3DG	−	−	−	−	+	+	+	−	+	−[f]

[a] Physiological groups 1, 2, 3, and 1-2 are equivalent to biovars 1, 2, 3 and 1-2 (6, 35).
[b] Reference strain for that physiological group.
[c] Type strain of a group of *Agrobacterium* strains isolated from the Allison Savanna sharing the same phenotype.
[d] Number of strains in that phenotypic group (see footnote *b*).
[e] Nine of the fourteen strains gave the indicated result.
[f] Thirteen of the fourteen strains gave the indicated result.

were added to a minimal liquid medium containing either opine as the sole carbon and nitrogen source. *Agrobacterium* strain R3/84 utilized octopine, and *Agrobacterium* strain S2/84 utilized separately both nopaline and octopine. The other opine utilizers were gram-negative bacteria which failed to produce a fluorescent pigment on King medium B; seven of these strains were oxidase positive.

DISCUSSION

Endemic nature of *Agrobacterium* spp. *Agrobacterium* spp. are endemic to the Allison Savanna and appeared to be a natural component of the soil microbiota. Large populations of agrobacteria were detected in all soil and root samples examined. The population levels in the soil were higher than has been our experience for cultivated soils. This is the first reported evidence for the presence of *Agrobacterium* spp. in undisturbed, nonagricultural soils, suggesting that this organism is not only a successful invader but also a true inhabitant of the midwest savanna soils. Furthermore, the data show that *Agrobacterium* spp. can reside on the roots of native plants, including an unidentified monocot within the family Poaceae.

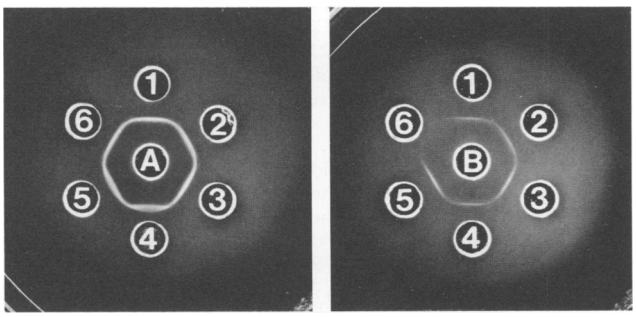

FIG. 1. Immunodiffusion patterns of ribosomal antisera to *Agrobacterium* spp. against strains isolated from the Allison Savanna. The center wells of panels A and B contain antiserum, respectively, to *Agrobacterium* strain M63/79 and *Agrobacterium* strain U11. Outer wells of panel A contain ribosomes extracted from strains M63/79 (wells 1 and 4), R6/84 (group 1) (well 2), S2/84 (group 2) (well 3), S4/84 (group 4) (well 5), and R5/84 (group 1-2) (well 6). Outer wells of panel B contain ribosomes from strains U11 (wells 1 and 4), S4/84 (group 4) (well 2), R5/84 (group 1-2) (well 3), R6/84 (group 1) (well 5), and 3R26 (unidentified rhizosphere bacterium) (well 6).

Predominance of nonpathogenic agrobacteria. The inability of all 54 *Agrobacterium* strains to infect tomato seedlings, a relatively good indicator of pathogenicity (1), may be due to their host specificity (1). However, the majority of these strains are probably not pathogenic, because nonpathogenic agrobacteria largely predominate over pathogenic agrobacteria in cultivated soils and the rhizosphere of healthy host plants (30, 33) and can even prevail in crown gall tumors (4). This overwhelming predominance of nonpathogenic agrobacteria underscores the need to understand their role in the disease cycle. Are these nonpathogenic agrobacteria in a form which would have pathogenicity genes (oncogenicity, virulence, or host specificity genes) repressed until triggered by favorable environmental conditions, thus transforming this otherwise saprophyte into an active pathogen? Alterna-

TABLE 3. Number of strains using nopaline or octopine or both as the sole carbon and nitrogen source in solid or liquid media

Bacteria	No. of strains using:					
	Nopaline only		Octopine only		Both opines[a]	
	S[b]	L[c]	S	L	S	L
Agrobacterium physiological group 1-2			1			
Agrobacterium physiological group 2			4	1	1	1
Nonfluorescent oxidase-positive	6	3	5	4	1	
Nonfluorescent oxidase-negative	5	1	6	2	1	

[a] These strains grow on either octopine or nopaline.
[b] Solid medium.
[c] Liquid medium.

tively, are these bacteria saprophytes until a pTi infects them?

Characteristics of naturally occurring agrobacteria. *Agrobacterium* biovar 2 strains predominated in this study and consisted of three phenotypically distinct groups. The largest group of biovar 2 strains isolated had the peculiar characteristic of not metabolizing erythritol, the carbon source used to selectively isolate biovar 2 agrobacteria (6, 29). The four biovar 1-2 strains differed from the representative biovar 3 strain CG64 in their utilization of erythritol and hence their growth on medium 2E. The 14 strains making up the fourth physiological group shared characteristics of both biovars 2 and 3. Members of this group did not grow on medium 3DG and differed from biovar 3 in their oxidase reaction, their ability to use erythritol, and their inability to utilize L-(+)-tartaric acid; they differed from biovar 2 in their inability to utilize L-tyrosine, mucic acid, L-(+)-tartaric acid, and malonate.

A problem confronted in this study, as well as in that of Spiess et al. (36), is how to identify nonpathogenic agrobacteria which do not fit in the accepted biovar groupings (16). However, we were able to identify the savanna strains to the genus level by using antisera to 50S ribosomal subunits of *Agrobacterium* spp. and our limited nodulation assays.

The existence of new physiological groups should not be surprising, because reports of pathogenic *Agrobacterium* strains that do not fit the assigned biovar are common (2, 10, 13, 26, 37; A. R. Anderson, Ph.D. thesis, Oregon State University, Corvallis, 1978). Future searches for *Agrobacterium* spp. in new habitats outside nurseries and orchards will probably demonstrate the ubiquitous nature of this group of organisms, their adaptation to different environments, and the presence of new biovars. Care should be taken not to rely solely on selective media in this search, because the new biovars may not grow on these media.

Preponderance of nonagrobacteria among opine utilizers. Opines, which are chemicals synthesized specifically by plant tissue genetically transformed by the pTi of *Agrobacterium* spp., are described as mediators of parasitism in the interactions between the pTi, its *Agrobacterium* host, and the tumorigenic plant cells (38). In this context, the bacterium harboring a pTi induces the development of a crown gall tumor, which is a specific ecological niche for itself, rich in the opine that only it can metabolize, favoring its growth and propagation (11). However, it has now been established that opine utilization is not confined to pathogenic agrobacteria. Strains of nonpathogenic agrobacteria (18, 24, 25, 27) and fluorescent pseudomonads (2, 6, 24, 31; L. W. Moore, Fourth Int. Congress Phytopathol. abstr. no. 141, 1983) were reported to utilize opines. In the present study, 30 of 126 strains isolated from soil and root samples grew on solid medium containing opines as the sole carbon and nitrogen source, but only 12 utilized opines in liquid medium. This demonstrates that the use of solid medium should be limited to rapid screening of opine utilizers. The fact that 83% of the opine utilizers were neither agrobacteria nor fluorescent pseudomonads clearly indicates that a large and diverse microbiota can utilize opines. Apparently, a pathogenic *Agrobacterium* strain faces a stronger competition in the opine environment it induced than was previously thought. The present isolation of opine utilizers from an environment a priori free of crown gall must lead us to rethink the origin and role of opines in the terrestrial environment and determine the ecological significance of opine utilization by bacteria which are not agrobacteria.

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