APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1990, p. 2576–2579
0099-2240/90/082576-04$02.00/0

Vol. 56, No. 8

Characterization of the Opine-Utilizing Microflora Associated with Samples of Soil and Plants

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Received 13 March 1990/Accepted 19 May 1990

Microorganisms utilizing an opine as the sole carbon source were recovered from crown gall tumors, soil, and surface-disinfected potato tubers. The effect of the opines octopine, nopaline, succinamopine, and mannopine as selective substrates was compared with that of the auxin indoleacetic acid. Selection on octopine and indoleacetic acid favored the fluorescent pseudomonads, whereas mannopine allowed the frequent recovery of agrobacteria. Coryneforms which utilized succinamopine or mannopine were detected in soil, but not in tumors. Fungi growing on succinamopine or mannopine and a mannopine-utilizing *Pseudomonas putida* were isolated from tumor and soil, respectively.

Opines are plant metabolites synthesized following the transfer of DNA from a phytopathogenic bacterium, *Agrobacterium tumefaciens*, to cells of susceptible plant hosts. The transferred DNA (T-DNA) exists within bacteria as part of the Ti plasmids and in the host plant cell as a set of expressed genes covalently integrated in the genome. Transfer of the T-DNA depends on the expression of the *chv* genes encoded by the chromosome and of the *vir* genes present on the Ti plasmids. Following their transfer, the genes of bacterial origin determine the neoplastic growth of transformed host tissue, resulting in the formation of a crown gall tumor and the synthesis of opines. Other genes on the Ti plasmids, which are not transferred, confer on the bacterium the ability to utilize opines for growth (2).

In addition to virulent agrobacteria, some avirulent agrobacteria, fluorescent and nonfluorescent pseudomonads, and coryneforms exhibit the ability to grow on opines as the sole carbon source (3, 15). The objective of the present study was to isolate as many different opine-utilizing bacteria as possible from a given sample of soil or plant tissue and also to compare, during these isolations, the selective pressure generated by opines with that imposed by another compound of plant origin, the auxin indoleacetic acid.

Bacteria were obtained from three different types of samples: two pear crown gall tumors and one soil sample, both collected at a nursery in Rougemont, Quebec, Canada, and potato tubers. Tumors and soil were treated as described before, with the in situ enrichment procedure being used for soil (15).

Eight potato tubers were sampled: two had been purchased at a local market, three were of cultivar Kennebec, and three were of cultivar Superior. A tuber was surface disinfected in 10% (vol/vol) commercial bleach and flamed on both sides with 70% (vol/vol) ethanol. A fragment of about 0.5 cm^3, comprising a section of both the surface and the vascular ring, was excised from the stem end. This tissue was crushed in 0.5 ml of saline. The resulting slurry was used to inoculate the selective media as described below.

Some of the selective media were adjusted to a final pH of 7.0 and contained AT salts (9) and 1 g of ammonium sulfate, 0.1 mg of biotin, and 100 mg of cycloheximide per liter. The test substrate was present at 800 mg/liter, except for succinamopine, consisting of a 50:50 mixture of the DL- and LL-isomers, which was added at 1.6 g/liter. When required, the following inhibitors were also added to the selective media: a combination of 400 mg of cefotaxime and 100 mg of lysozyme per liter; a combination of 100 mg of nitrofurantoin and 2 mg of crystal violet per liter; 100 mg of nitrofuantoin per liter alone; 2 mg of crystal violet per liter alone; and a combination of 200 mg of carbenicillin and 2 mg of crystal violet per liter.

A second set of selective media had a final pH of 5.8. The composition was as stated above for the media adjusted to pH 7.0, except that the regular AT salts were replaced with a modified solution, called AT-M. This contained 15 g of KH_2PO_4 per liter instead of 10.9 g and was adjusted to a pH of 5.8 with KOH. The pH 5.8 media were prepared with or without 240 mg of vanillin per liter.

To initiate primary cultures in the selective media, 0.1 ml of inoculum prepared from crown gall tumors, preenriched soil, or potato tubers was transferred to 1 ml of medium.

Primary cultures were incubated at 27°C with shaking for up to 14 days, and a loopful from cultures showing evidence of bacterial growth was transferred to nutrient agar (Difco Laboratories, Detroit, Mich.) containing 200 mg of cycloheximide per liter. Primary cultures showing signs of fungal growth were vortexed, and a loopful of culture was transferred to potato dextrose agar (Difco) adjusted to pH 3.5 with tartaric acid. Bacteria and fungi were further purified on solid medium.

Utilization of opines by the isolates was verified as described previously (15). Utilization of indoleacetic acid was confirmed following growth by colorimetric analysis of the supernatant (8). Standard determinative tests were done as described previously (15). Calcofluor staining of colonies was examined by the method of Cangelosi et al. (5). The API Rapid NFT profile was determined as specified by the supplier, API Laboratory Products Ltd., St. Laurent, Quebec, Canada. SLS and SDS medium were based on nutrient agar (Difco) and also contained 1.2 g of sodium lauroyl sarcosine (for SLS medium) or sodium dodecyl sulfate (for SDS medium) per liter. Medium IA (selective for *Agrobacterium* biotype 1) and medium 2E (selective for *Agrobacterium* biotype 2) were those of Brisbane and Kerr (4).

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TABLE 1. Reactions of the various types of bacterial isolates to diagnostic tests and hybridizations

Test or hybridization	Bacterial type					
	Fluorescent *Pseudomonas* spp. (n = 30)	*Agrobacterium* biotype 1 (n = 23)	*Agrobacterium* biotype 2 (n = 5)	*Agrobacterium* spp. (unknown biotype) (n = 2)	Coryneform bacteria (n = 7)	Other gram-negative bacteria (n = 22)[a]
Gram stain	−	−	−	−	Variable	−
Lysis in KOH	+	−	−	+	−	+
Fluorescence on King medium B	+	−	−	−	−	−
3-Ketolactose production	−	+	−	−	−	−
Growth in 1.2 g of sodium lauroyl sarcosine per liter	+	+	+	−	−	+
Growth in 1.2 g of sodium dodecyl sulfate per liter	+	−	−	−	−	+
Growth in *Agrobacterium* selective medium 1A	−	+	D[b]	−	−	−
Growth in *Agrobacterium* selective medium 2E	−	D	+	−	−	−
Oncogenicity	−	−	−	−	−	−
Fluorescence on calcofluor	−	+	+	+	NT[c]	−
Hybridization to *chvA* and *chvB* gene probes	−	+	+	+	NT	−
Hybridization to *virA*, *virB*, and *tms2* gene probes	−	−	−	−	NT	−

[a] Includes nonfluorescent pseudomonads and other isolates which could not be identified.
[b] D, Positive or negative reactions to this test were observed, depending on the isolate.
[c] NT, Not tested.

Bacteria were introduced into plants by wounding the stem with a needle previously dipped in a bacterial colony. Inoculated plants were maintained in the greenhouse for 5 to 6 weeks. All bacterial isolates were tested for oncogenicity on tomato (cv. Vendor), sunflower (cv. Russian Mammoth), tobacco (cv. Xanthi nc), and *Kalanchoe daigremontiana*. The gram-negative bacteria were further tested on *Nicotiana rustica*, pumpkin (cv. Hybrid Spirit), and squash (hybrid F1 zucchini). *A. tumefaciens* C58 and B6S3 served as positive controls.

The probe for the *chvA* gene of *A. tumefaciens* C58 was a 2.4-kilobase (kb) *Hin*dIII fragment cloned as pGDS28 into pACYC177, and the *chvB* gene probe was a 1.2-kb *Eco*RI fragment cloned as pTY19 into pUC19 (7). The probes for pTiC58 genes *virB* and *virD* were *Bam*HI fragments 23, of 2.1 kb, and 27, of 1.7 kb, respectively. The probe for the T-DNA gene *tms2* from pTiC58 consisted of 2.2-kb *Hin*dIII fragment 22 (11). These restriction fragments from pTiC58 were contained in cosmid clones obtained by other authors (10). Total genomic DNA from the isolates was digested to completion with *Eco*RI. DNA samples (5 μg) were electrophoresed, blotted, and hybridized under high-stringency conditions to probes labeled with [^{32}P]dCTP (12).

The microorganisms considered in this study grew within 1 week or less on the test substrate which had been used for their isolation. Growth was accompanied by complete utilization of the test substrate.

None of the bacterial isolates fermented glucose. Bacteria were grouped according to distinctive reactions to diagnostic tests (Table 1). Some of the gram-negative isolates which could not be identified on the basis of the diagnostic tests listed in Table 1 were classified as nonfluorescent pseudomonads, using the API Rapid NFT system. The nonfluorescent, mannopine-utilizing isolate NA513, which originated from soil, was identified as *Pseudomonas putida* by the American Type Culture Collection, Rockville, Md. Other isolates were indistinguishable from NA513 on the basis of the tests performed here.

Octopine and indoleacetic acid were substrates very selective for fluorescent pseudomonads, and no agrobacteria were recovered on these compounds. Bacterial populations obtained on the other three substrates did include some agrobacteria. In contrast with the results of the octopine selection, mannopine-utilizing fluorescent pseudomonads were not obtained. Differences were found between the populations extracted from either tumors or soil. Fungi utilizing succinamopine or mannopine were isolated from one of the pear tumors. Coryneform bacteria were obtained from soil only. The use of mannopine as a selective substrate allowed the recovery of a homogeneous bacterial population from the two tumor samples, since only biotype 1 and 2 agrobacteria were recovered. In contrast with this, mannopine-utilizing bacteria as extracted from soil were more diverse. Opine-utilizing bacteria were recovered from three of eight potato tubers sampled (Table 2).

The addition of various inhibitors to the opine-containing selective media made possible the recovery of succinamopine-utilizing fluorescent pseudomonads and fungi growing on succinamopine or mannopine, as these two types of microorganisms were not recovered on regular medium without inhibitors (results not shown).

Octopine, nopaline, succinamopine, and mannopine can be assembled in combinations which are not usually found within the same crown gall tumor (13). Nevertheless, these four opines were substrates for bacteria colonizing the two pear tumors sampled in this study. Similarly, potato tubers are made of untransformed tissue and hence are not expected to synthesize any opine. Once again, opine utilizers with various catabolic abilities were isolated from some of the surface-disinfected tubers tested.

The agrobacterial isolates were unable to induce tumors upon inoculation to various host plants. They fluoresced in the presence of calcofluor, a phenotype conferred on strains of *Agrobacterium* and *Rhizobium* spp. by exopolysaccharide synthesis (5). They also showed homology to probes representing chromosomal genes, *chvA* and *chvB*, which are responsible for the synthesis and secretion of the cyclic β-1,2,-glucan typical of bacteria of the family *Rhizobiaceae* (5). However, no homology was found between the total DNA from these agrobacterial isolates and probes derived from the Ti plasmid-encoded genes *virB*, *virD*, and *tms2*. Whereas some oncogenic strains of *A. tumefaciens* were found also to lack homology to the T-DNA-encoded *tms2* gene (16), the nontransferred *virB* and *virD* genes are con-

TABLE 2. Recovery and identification of opine-utilizing isolates

Substrate used for isolation	Identification[a]	No. of isolates	Origin
Octopine	Fluorescent pseudomonads	4	Pear tumor no. 1
		3	Pear tumor no. 2
		3	Soil
		3	Potato tuber (market)
	Nonfluorescent pseudomonads[b]	1	Potato tuber (Kennebec no. 1)
		1	Potato tuber (Kennebec no. 2)
Nopaline	Fluorescent pseudomonads	1	Pear tumor no. 1
		2	Pear tumor no. 2
	Nonfluorescent pseudomonads	1	Pear tumor no. 2
		1	Potato tuber (Kennebec no. 1)
	Agrobacterium biotype 1	1	Pear tumor no. 1
	Coryneform bacterium	1	Potato tuber (Kennebec no. 2)
Succinamopine	Fluorescent pseudomonads	1	Pear tumor no. 1
		2	Pear tumor no. 2
		4	Soil
	Nonfluorescent pseudomonads	2	Pear tumor no. 1
	Agrobacterium biotype 1	4	Pear tumor no. 1
		1	Pear tumor no. 2
	Agrobacterium sp., unknown biotype	2	Potato tuber (market)
	Coryneform bacterium	1	Soil
	Fungi	3	Pear tumor no. 2
Mannopine	*P. putida*[c]	6	Soil
	Agrobacterium biotype 1	4	Pear tumor no. 1
		9	Pear tumor no. 2
		3	Soil
		1	Potato tuber (Kennebec no. 2)
	Agrobacterium biotype 2	4	Pear tumor no. 1
		1	Soil
	Coryneform bacteria	4	Soil
		1	Potato tuber (Kennebec no. 1)
	Fungus	1	Pear tumor no. 2
Indoleacetic acid	Fluorescent pseudomonads	2	Pear tumor no. 1
		2	Pear tumor no. 2
		3	Soil

[a] Bacteria which could not be identified in the course of this study are not included. Unless stated otherwise, identifications are based on the determinative tests listed in Table 1.

[b] Identification of bacteria listed as nonfluorescent pseudomonads is based on the API Rapid NFT profile.

[c] Identification provided by the American Type Culture Collection.

sidered essential for virulence (2, 14), and sequences homologous to these genes are carried by narrow-host-range strains (16). Thus, the lack of homology to the *vir* genes confirms the avirulent character of the agrobacterial isolates.

Opine-utilizing bacteria are recovered effectively with liquid media, but this efficiency may be at the expense of diversity within the final collection of isolates (15). To alleviate this problem, sets of parallel cultures were prepared from the tumor and soil samples, cultures from any given set being presented with the same selective substrate and a variety of inhibitors. This strategy allowed the recovery of some microorganisms, particularly fungi, which were not detected in the absence of inhibitors. In a separate study, the fungal isolates were identified as *Cylindrocarpon* and *Fusarium* spp. (1).

The ability to utilize mannopine is uncommon, though not absent, among natural pseudomonad populations. In a previous study, Ti plasmid-encoded genes of mannopine catabolism were transferred to a strain of *P. fluorescens* and expressed (6).

Some tumor-derived substrates, such as the auxin indoleacetic acid and the opine octopine, appear of little selective value for agrobacteria. However, the synthesis by crown gall tumors of mannopine, a compound generally not utilized for growth by pseudomonads, would confer a nutritional advantage on agrobacteria. Results obtained here and in a previous study (15) suggest that coryneform bacteria are excluded from crown gall tumors. Mannopine-utilizing fungi may be expected to intervene only at a late stage of tumor colonization. Possibly, virulent and avirulent agrobacteria have exclusive access to the mannopine produced by the tumors.

We thank Normand Arcand, Abd-Allah Mba-Ostagne, and Denise Auclair for assistance. We are grateful to Gilbert Banville, Donald R. Helinski, Stephen K. Farrand, and W. Scott Chilton for supplying potato tubers, clones, and opines.

This work was supported by grants from the Conseil des Recherches en Pêche et Agro-Alimentaire du Québec (2083 and 2084), the Natural Science and Engineering Research Council of Canada (A-7640), and Ciba-Geigy Corp.

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