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Diversity among Opine-Utilizing Bacteria: Identification of Coryneform Isolates

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Bacteria were isolated from soil and crown gall tumors by selection in minimal medium with an opine, such as succinamopine or mannopine, as the sole carbon source. The isolates were characterized for the pattern of opine utilization and identified. They were classified as mannityl opine or imino diacid utilizers and exhibited specificity of utilization similar to that described previously for *Agrobacterium* species. A minority of isolates were gram negative and were identified as *Agrobacterium* or *Pseudomonas* species; most were gram positive and belonged to the coryneform group. These results indicate that any specific effect of opines on the ecology of *Agrobacterium tumefaciens* is modulated by activities of other types of soil- and plant-associated bacteria.

Infection of susceptible plants with virulent cells of *Agrobacterium* results in the development of crown gall tumors and the synthesis of new metabolites called opines. The genes of opine synthesis are carried by bacterial Ti or Ri plasmids and are transferred to the plant cells in which they become expressed. Other plasmid genes that are not transferred enable *Agrobacterium* species to utilize opines as the sole carbon source, nitrogen source, or both (3, 21). The various opines have been grouped into families according to chemical structure; for example, octopine, octopinic acid, nopaline, succinamopine, and leucinopine are classified in the imino diacid family; mannopine, mannopinic acid, and agropinic acid belong in the mannityl opine family (7, 9). The particular opines that are found in a given crown gall tumor correspond to those that can be catabolized by the inciting bacteria (23).

Unraveling of the genetic relationships involved in opine metabolism raised the hypothesis that the utilization of opines was a unique feature of virulent agrobacteria and closely related strains. This hypothesis was then developed into the opine concept, stating that agrobacteria gain a selective advantage from opine synthesis by transformed plant cells (29). This concept should be revised to take account of the numerous reports of opine utilization by pseudomonads, however (1, 5, 19, 20, 25; C. Pootjes, A. Montoya, and E. Nester, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, K75, p. 139; W. Beckman and T. G. Lessie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, H112, p. 132; M. L. Canfield, J. Boe, and L. W. Moore, Phytopathology **74**:1136, 1984).

As a contribution to the systematic evaluation of diversity among opine-utilizing bacteria, various types of opines were used as the selective substrate in cultures initiated from crown gall tumors or soil samples. Previous isolation studies often involved the use of media that were selective for *Agrobacterium* species and thus maintained a taxonomic bias (1, 25). Furthermore, these early studies concentrated on three opines, octopine, octopinic acid, and nopaline. In this study no deliberate bias was introduced and other opines, particularly mannopine and succinamopine, were presented to the bacteria. Many of the isolates thus obtained were gram-positive bacteria, whereas to our knowledge the capacity for opine utilization had, until now, solely been recognized in gram-negative species.

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MATERIALS AND METHODS

Isolation of opine-utilizing bacteria. Bacteria described in this study were isolated from samples of soil and apple crown gall tumors. Soil samples were of two different types: soil from land with crops of potatoes or cereals and collected from Les Buissons, Pointe-aux-Outardes, north of Quebec City (these samples are collectively designated as originating from the northern field) and soil from three apple tree nurseries located in southeast Quebec, either at Rougemont for two commercial nurseries or at Frelighsburg for an experimental nursery operated by Agriculture Canada. Apple crown gall tumors were collected from Malling 9 and Malling 26 apple rootstocks and a standard apple seedling.

Two methods, direct selection and in situ enrichment, were used for isolation. For direct selection, unsieved soil (1 g) was suspended in 10 ml of saline (8.5 g/liter of NaCl), while apple tumor samples (0.1 g) were cut into small pieces and soaked for 60 min in 10 ml of saline. These suspensions were diluted 100-fold in saline, and 0.1 ml from each of the dilute suspensions was transferred to 1 ml of cycloheximide-opine medium with AT salts (15) and the following, per liter: 1 g of $(NH_4)_2SO_4$; 100 mg of cycloheximide; 0.1 mg of biotin; 10 mg each of nicotinic acid and calcium pantothenate; and finally, 800 mg of the test opine, as indicated. The cultures were incubated for 5 days at room temperature with shaking at 175 rpm on a model G33 New Brunswick rotary shaker. Samples from growing cultures were then streaked for single colonies onto nutrient agar (Difco Laboratories, Detroit, Mich.) containing 200 mg of cycloheximide per liter and purified by successive transfers on this medium. Purified isolates were then retested for opine utilization by inoculation in 1 ml of medium similar to that described above for the cycloheximide-opine medium, but without the antibiotic. The initial optical density at 600 nm was 0.01, and the bacteria were incubated for 4 to 5 days; if positive (final optical density, 0.2 or more) they were stored in 15% glycerol at −75°C.

In situ enrichment allowed for the amplification of presumptive opine-utilizing populations that were then readily detected. Cycloheximide-opine medium (2 ml; see above)

was added to 10 g of soil, and the mixture was incubated for 8 days at 25°C in the dark. Saline (10 ml) was then added and the resulting slurry was shaken vigorously. A 100-fold dilution was prepared in saline, and 0.1 ml of the dilution was transferred to 1 ml of the cycloheximide-opine medium. The procedure then followed was that described above for the direct selection method.

Diagnostic tests. The isolates were observed under a phase-contrast microscope to detect endospores. The capacity for survival to 60°C was verified as indicated by Jones (16). The ability to fix nitrogen was tested by incubation for 10 days in modified Burk medium (26). The Ziehl-Neelsen method (14) was used for acid-fast staining. Chitinase production was tested by incubating bacteria for 7 weeks in a medium containing AT salts and the following, per liter: 1 g of NH_4Cl, 5.5 g of yeast extract, 2 g of chitin, and 15 g of agar. For Gram staining, the reagents from Difco were used according to the instructions of the manufacturer. The procedure described by De Ley and Rassel (13) was used for observing flagella by electron microscopy, except that phosphotungstic acid was prepared at 1% (wt/vol; pH 6.0) and the washing step in distilled water was omitted. For gelatin hydrolysis, both a liquefaction method and a method involving detection of hydrolysis zones with $HgCl_2$ were used; the second method is reported to be more sensitive than the first (26). Growth in the presence of 5% (wt/vol) NaCl was tested by adding salt to nutrient broth medium (Difco) or to minimal medium with AT salts; 2 g of glucose per liter; 1 g of $(NH_4)_2SO_4$ per liter; and biotin, calcium pantothenate, and nicotinic acid, as described above. The cultures were examined after 1 week, and the occurrence of growth in one or both media was recorded as a positive reaction. Other tests were done by the method described by Smibert and Krieg (26) or as described in Table 2.

Oncogenicity. The capacity of the isolates to induce tumors was tested on tomato (*Lycopersicon esculentum* cv. Vendor), sunflower (*Helianthus annuus* cv. Russian Mammoth), tobacco (*Nicotiana rustica*), Kalanchoe (*Kalanchoe daigremontiana*), and apple (*Malus pumila* cv. Cortland). Bacteria were grown on nutrient agar (Difco) for 24 to 48 h, and 4- to 8-week-old plants were wound-inoculated at three different internodes with a needle that had been dipped in a bacterial colony, except for apple, which was inoculated only once at the crown. The inoculated plants were kept under standard greenhouse conditions for 4 weeks (8 weeks in the case of apple) and examined.

Cell wall analysis. Cell wall preparations were obtained by the Aberdeen method as described by Bousfield et al. (4). These preparations were then subjected to the hydrolysis recommended by these investigators for amino acid or sugar analysis. Amino acids were separated by thin-layer chromatography; plates coated with cellulose (no. 13255; Eastman Kodak Co., Rochester, N.Y.) were developed 3 times with isopropanol-formic acid-water (40:2:10) (24), and the amino acids were revealed with ninhydrin (4). Sugar analysis was done by paper chromatography (4).

Utilization of opines. Bacteria were inoculated in Bergersen (2) liquid medium supplemented with 1 g of $(NH_4)_2SO_4$ per liter, 400 mg of mannitol per liter, and a combination of opines at 1 g/liter each. Two cultures were prepared for each isolate. In the first culture mannopine and DL-nopaline were added together as the test substrates, while for the second agropinic acid, LL-nopaline, DL-octopine, DL-succinamopine, and LL-succinamopine were presented. The cultures were incubated at 25°C with shaking. At intervals, a portion from each culture was centrifuged and analyzed for opine contents by paper electrophoresis, as described previously (7, 10).

With the exception of LL-nopaline, all the opines used in this study are known to occur in at least some types of crown gall tumors.

Chemicals. Opines were prepared by chemical synthesis as described previously (7–10). In this report, the absolute configuration of the imino diacid opines is designated by reference to the chirality of the component amino acids. For example, natural nopaline is the *erythro* isomer with the D-glutamyl and the L-arginyl configuration and is referred to, for brevity, as DL-nopaline.

RESULTS

Isolation of opine-utilizing bacteria and characteristics of opine utilization. In total, 33 opine-utilizing isolates were obtained (Table 1). By using direct selection on mannopine, leucinopine, mannopinic acid, or agropinic acid, bacteria were recovered from 5 of the 17 soil samples tested and 2 of the 3 tumor samples tested. Introduction of an in situ enrichment step before selection in liquid medium facilitated the recovery. In a comparison of the two methods, two samples from commercial nursery 1 and three from northern field were considered. With succinamopine as the selective substrate, only one sample from each of these two locations yielded bacteria following direct selection, whereas all of the attempts to yield bacteria were successful with in situ enrichment. With mannopine no bacteria were recovered by direct selection, while with in situ enrichment attempts were successful for all the samples but one originating from commercial nursery 1.

The isolates readily utilized as the sole carbon source the particular opine on which they were selected. In most cases the maximal optical density was reached within 72 h and was greater than 0.2. Growth was less abundant (maximal optical density, about 0.1) for the isolates that were obtained on LL-leucinopine and retested on this compound; however, these bacteria were still considered positive for opine utilization because leucinopine is a poor substrate, at least for *Agrobacterium* species (8).

The bacteria were also tested for catabolism of seven different opines (Table 1). A small amount of mannitol, a carbon source that was readily utilized by all the isolates except GU28 and GU36 (data not shown), was included in these experiments to ensure rapid initial growth. About half of the isolates utilized another opine or more, in addition to that on which they were selected. Catabolism of those additional substrates usually occurred either more slowly than that of the selective opine or at a similar rate. Isolate GU36 was an exception, because DL-nopaline was catabolized faster than the DL-succinamopine that was used in isolation. The capacities for catabolism of mannityl opines and imino diacids remained dissociated in most of the isolates, except for two that utilized mannityl opines and LL-nopaline. Some of the imino diacid-utilizing bacteria catabolized up to three different opines but always exhibited a strict specificity with respect to the stereochemistry of the substrate.

Variability was observed in the time required by some isolates for complete catabolism of imino diacids other than the one used for isolation; for example, on several occasions isolate GU17 utilized DL-nopaline after 50 h, whereas in other experiments it did not utilize this opine at all. In one experiment not included in Table 1, the imino diacids DL-nopaline, DL-octopine, DL-succinamopine, and LL-suc-

TABLE 1. Bacteria isolated in this study

Taxonomic identity	Isolate designation	Origin	Selective substrate used for isolation	Isolation method[a]	Opine completely catabolized after[b]: 50 h	120 h	190 h
Agrobacterium biotype 1	GU10, GU11	Apple tumor, commercial nursery 1	Mannopine	DS	MOP, AGA		
Agrobacterium biotype 2	GU7	Apple tumor, commercial nursery 2	Mannopine	DS	MOP, AGA		
	GU31	Soil, commercial nursery 1	Mannopine	IE	MOP, AGA		
Pseudomonas fluorescens	GU24	Soil, commercial nursery 1	Succinamopine[c]	IE	LL-SAP		
Pseudomonas putida	GU28	Soil, commercial nursery 1	Succinamopine	IE	DL-SAP	DL-NOP	
Coryneform subgroup A	GU2	Soil, commercial nursery 2	Mannopine	DS	MOP, AGA		
	GU9	Sand, commercial nursery 2	Agropinic acid	DS	MOP, AGA		
	GU13	Soil, northern field	Mannopine	IE	MOP, AGA	(LL-NOP)	
	GU32	Soil, northern field	Mannopine	IE	MOP, AGA, (LL-NOP)		
	GU16	Soil, commercial nursery 1	Mannopine	IE	MOP, AGA		
	GU17	Soil, northern field	Succinamopine	IE	DL-SAP, (DL-NOP)	(DL-OCT)	
	GU18	Soil, northern field	Succinamopine	IE	DL-SAP	DL-OCT	
	GU19	Soil, northern field	Succinamopine	IE	DL-SAP	(DL-OCT), (DL-NOP)	
	GU21	Soil, northern field	Succinamopine	IE	DL-SAP	DL-NOP	DL-OCT
	GU22	Soil, northern field	Succinamopine	IE	DL-SAP, DL-NOP		DL-OCT
	GU27	Soil, northern field	Succinamopine	IE	DL-SAP		
	GU20	Soil, commercial nursery 1	Succinamopine	IE	DL-SAP		DL-NOP, DL-OCT
	GU29	Soil, commercial nursery 1	Succinamopine	DS	DL-SAP		
	GU30	Soil, northern field	Mannopine	IE	MOP		
	GU33	Soil, northern field	Mannopine	IE	MOP		AGA
	GU35	Soil, commercial nursery 1	Succinamopine	IE	DL-SAP	(DL-NOP)	
	GU36	Soil, northern field	Succinamopine	DS	DL-NOP	DL-SAP (DL-OCT)	
Coryneform subgroup B	GU23	Soil, northern field	Succinamopine	IE	DL-SAP		(DL-NOP)
	GU26	Soil, northern field	Succinamopine	IE	DL-SAP		
	GU25	Soil, commercial nursery 1	Succinamopine	IE	LL-SAP	LL-NOP	
Coryneform subgroup C	GU15	Sand, Agriculture Canada nursery	Mannopinic acid	DS		MOP	
Coryneform subgroup D	GU4	Soil, commercial nursery 1	LL-Leucinopine	DS	LL-SAP	(LL-NOP)	
	GU14	Soil, commercial nursery 1	LL-Leucinopine	DS	LL-SAP	LL-NOP	
	GU8	Sand, commercial nursery 2	Mannopine	DS	MOP, AGA		
Coryneform subgroup E	GU3	Soil, commercial nursery 1	Mannopine	DS	MOP	AGA	
Coryneform subgroup F	GU1	Sand, Agriculture Canada nursery	Mannopine	DS	MOP		
	GU5	Soil, commercial nursery 2	Mannopine	DS	MOP, AGA		

[a] Abbreviations: DS, Direct selection; IE, in situ enrichment.

[b] Abbreviations: AGA, Agropinic acid; MOP, mannopine; DL-SAP, DL-succinamopine; LL-SAP, LL-succinamopine; DL-NOP, nopaline; LL-NOP, iso-nopaline; DL-OCT, octopine. Some isolate-opine combinations yielded variable results over several independent cultures; in those cases complete catabolism is indicated in parentheses at the time when it was most frequently observed.

[c] The succinamopine used in the isolations was a 50:50 mixture of DL and LL isomers.

TABLE 2. Distinctive characters for the various groups of opine-utilizing isolates

Test	Reaction of the various groups				Reference for test[a]
	Agrobacterium spp.		Fluorescent pseudomonads	Coryneforms	
	Biotype 1	Biotype 2			
Gram stain	−	−	−	Variable	
Oxidase	+	−	+	−	
Fluorescence on King medium B	−	−	+	−	27
Accumulation of PHB granules[b]	+	+	−	−	
Flagellar presence and arrangement	Peritrichous	Peritrichous	Polar	E[c]	
Growth on Brisbane and Kerr selective media:					5
1A	+	−	−	−	
2E	−	+	−	−	
3DG	−	−	−	−	
Production of 2-ketolactose	+	−	−	−	22
Acid in litmus milk	−	+	−	−	
Esculin hydrolysis	+	+	−	+	
Arginine dihydrolase	−	−	+	−	27
Urease	+	+	−	+	11
Denitrification	−	−	D[d]	−	27
Gelatin hydrolysis (liquefaction of gelatin)	−	−	D	+	
Gelatin hydrolysis (detection with $HgCl_2$)	+	−	D		
Casein hydrolysis	−	−	D	+	
Starch hydrolysis	−	−	−	E	
DNA hydrolysis	−	−	−	E	
Growth in 5% NaCl	−	−	−	E	

[a] Tests not considered in this column were done as recommended by Smibert and Krieg (26) or as indicated in the text.
[b] PHB, Poly-β-hydroxybutyrate.
[c] E, The coryneform isolates differed in their reaction to this test; the individual reactions for each of the isolates are given in Table 3.
[d] D, The two fluorescent pseudomonads showed a different reaction to this test; GU24 was positive, while GU28 was negative.

cinamopine were presented, either separately or with all four together, to isolates GU17, GU18, and GU19. For the three isolates, DL-nopaline was not catabolized after 120 h when present alone, but it was completely utilized after 52 h of incubation in the opine mixture.

Identification of the isolates. The opine-utilizing isolates shared many important characteristics. They were catalase positive and utilized glucose by oxidation; they produced H_2S from cysteine. They exhibited a negative reaction to the following tests: oncogenicity; the presence of endospores; survival to 60°C; nitrogen fixation; indole production; methyl red; Voges-Proskauer; acid-fast staining; Tween 80 hydrolysis; and finally, cellulase, chitinase, and phenylalanine deaminase activity. The isolates could be grouped into three broad categories, however, on the basis of reaction to the Gram stain and other diagnostic tests. Four of the gram-negative isolates were classified as *Agrobacterium* species and the two others as *Pseudomonas* species (Table 2).

The 27 other isolates were inconsistent in their reaction to the Gram stain, usually exhibiting a mixture of positive and negative cells irrespective of the growth stage. The gram-positive nature of the cell wall was confirmed by the observation that the cells did not lyse in KOH (28). Exponential-phase cultures in complex medium consisted of pleomorphic rods, sometimes with a rudimentary form of branching. Small coccoid cells were found in stationary-phase cultures. These characteristics indicate that the gram-positive isolates are coryneform bacteria. This was confirmed by reactions to several diagnostic tests (see above and Table 2) and demonstration of the absence of *meso*-diaminopimelic acid and arabinose in the cell wall, two compounds that are normal constituents of the bacterial cell wall but that are absent in some types of coryneforms (18). Further analysis showed that the isolates could easily be classified into six subgroups according to two criteria related to cell wall composition: type of diamio acid and occurrence of rhamnose. The isolates were also characterized further by means of conventional tests (Table 3).

DISCUSSION

Further demonstration of diversity among opine-utilizing bacteria. Earlier studies on opine utilization by various types of *Pseudomonas* species have already elicited comments on the diversity that exists among opine-utilizing bacteria (1, 25). Although some of the isolates obtained in this study were again identified as *Agrobacterium* or *Pseudomonas* species, the main interest of the results presented here stems from the novel nature of the coryneform bacteria. The isolation of these bacteria depended on three important features of this study. First, no deliberate taxonomic bias was introduced in the isolation protocols (see above). Second, samples with different natures and origins were analyzed. In particular, with mannopine as the selective substrate, agrobacteria were recovered from crown gall tumors, while isolations from soil samples yielded mainly coryneform bacteria. Third, a wide range of opines was presented as the selective substrate. In relation to this, it appears useful to summarize here the main conclusions from a separate study, in which soil and crown gall tumor samples were collected from the same nurseries from which samples were obtained for this study (see above). These samples were then assayed by the direct selection method for the presence of bacteria that utilize octopine, octopinic acid, or nopaline. Under these conditions gram-negative bacteria, identified as pseudomonads and agrobacteria, were recov-

TABLE 3. Subgroups of coryneform isolates

Subgroup	Type of diamino acid in wall	Rhamnose in wall	Isolate designation	Production of yellow nondiffusible pigment[a]	Flagellar presence and arrangement[b]	Hydrolysis of:		Growth in 5% NaCl
						Starch	DNA	
A	Lysine	−	GU2	−	Negative	−	−	−
			GU9	−	Negative	−	+	+
			GU13	−	Peritrichous	−	−	−
			GU16	−	Negative	+	−	−
			GU17	−	Negative	−	+	+
			GU18	+	Negative	−	+	+
			GU19	+	Negative	−	+	+
			GU20	+	Negative	+	+	+
			GU21	+	Peritrichous	−	+	+
			GU22	+	Peritrichous	−	+	+
			GU27	−	Negative	−	+	+
			GU29	−	Negative	−	−	+
			GU30	−	Peritrichous	−	−	−
			GU32	−	Peritrichous	−	−	−
			GU33	−	Negative	−	−	−
			GU35	+	Negative	−	+	+
			GU36	−	Negative	−	−	−
B	Lysine	+	GU23	−	Negative	−	+	+
			GU25	−	Negative	+	+	+
			GU26	−	Negative	−	+	+
C	Ornithine	−	GU15	−	Negative	−	−	+
D	Ornithine	+	GU4	−	Peritrichous	−	−	−
			GU8	−	Negative	−	+	−
			GU14	−	Peritrichous	−	−	−
E	Diaminobutyric acid	−	GU3	−	Negative	−	−	−
F	Diaminobutyric acid	+	GU1	−	Negative	+	+	−
			GU5	−	Negative	−	+	+

[a] Production of pigment was observed after incubation for 5 days on nutrient agar (Difco).
[b] Negative, absence of flagella; the term peritrichous refers to flagellar arrangement.

ered from 15 of the 21 soil samples tested and from all of the 6 tumor samples. No coryneform isolate has been obtained with octopine, octopinic acid, or nopaline (G. Tremblay, R. Lambert, H. Lebeuf, and P. Dion, Phytoprotection, in press). Thus, the various opines differ in the selective pressure that they impose when they are present as the sole carbon source.

There is no doubt that selection in liquid medium can lead to loss of the diversity that exists in the original soil or tumor population because of competition for nutrients and synthesis of inhibitors. Thus, it appears remarkable that other features of the isolation protocols (see above) still made possible the isolation of coryneforms, a new type of opine-utilizing bacterium. We speculate that the main reason for this success is that pseudomonads would generally be unable to catabolize the opines which were used in this study. Indeed, pseudomonads are often rapidly growing bacteria that produce a variety of inhibitors and are likely to be recovered whenever they occur in a mixed culture of soil bacteria.

The opine-utilizing coryneforms share many characters with representatives of the genus *Arthrobacter*, including the lack of a requirement for growth factors, nutritional versatility, and habitat (17). In the case of isolates belonging to subgroups A and B (see Table 3), cell wall composition also corresponds to that characteristic for *Arthrobacter* species (17, 18). Although the significance of purely morphological distinctions among the coryneforms has been questioned (16), the absence of a rod-coccus cycle in the gram-positive bacteria described in this study precludes the definitive identification of subgroups A and B isolates as members of the genus *Arthrobacter*. Further analysis, and particularly polyacrylamide gel electrophoresis of cellular proteins (6), would be needed to establish a firm identity.

A minority of coryneform isolates had ornithine or diaminobutyric acid in the cell wall. These bacteria do not belong in the genus *Arthrobacter*, because results of previous studies have emphasized the fundamental significance of cell wall composition in the taxonomy of the coryneforms (12, 18).

Common patterns of opine catabolism among the various bacterial types. Irrespective of taxonomic identity, most of the isolates obtained in this study could be classified as mannityl opine, DL-imino diacid, or LL-imino diacid utilizers. Thus, these results are consistent with the existence of three corresponding classes of catabolic enzymes (7, 9, 10) and suggest that most of the isolates possess enzymes of only one of these classes. Two exceptions would be strains GU13 and GU32, which were isolated from the same sample and showed very similar properties. These bacteria possess mannityl opine-catabolizing enzymes as well as an LL-imino diacid catabolase, a combination that is also found in *Agrobacterium* strains carrying plasmid pTiBo542 (9).

It has been shown that, while highly stereospecific, the enzymes responsible for opine catabolism in *Agrobacterium* species were relatively tolerant of side chain variation (7, 9, 10). A similar situation may be found here, in which some of the isolates selected for growth on leucinopine or suc-

cinamopine showed, to a variable extent, the ability to grow on related opines of the correct stereochemistry. Differences with respect to the number of opines that are catabolized by each isolate and the time required for this catabolism could possibly reflect variations in the capacity of the isolates to respond to different opines as inducers of the catabolic enzymes.

In complex natural environments opines are only one of the many components that settle the outcome of microbial competition. The results presented here lead us to conclude that this opine component does not determine whether gram-negative or gram-positive bacteria prevail in crown gall tumors or in soil. It would confer a selective advantage on opine-utilizing cells, however, once a particular bacterial type has become established.

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