Frequent occurrence of the ability to utilize octopine in rhizobacteria

CHANTAL J. BEAUCHAMP[1,2]

Département des sols, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Ste-Foy (Qué.), Canada G1K 7P4

JOSEPH W. KLOEPPER

Department of Plant Pathology, Alabama Agricultural Experiment Station, Auburn University, Auburn, AL 36849, U.S.A.

RAN LIFSHITZ

Beak Consultants Ltd., 14 Abacus Road, Brampton, Ont., Canada L6T 4B7

PATRICE DION

Département de phytologie, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Ste-Foy (Qué.), Canada G1K 7P4

AND

HANI ANTOUN

Département des sols, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Ste-Foy (Qué.), Canada G1K 7P4

Received March 19, 1990

Accepted September 21, 1990

BEAUCHAMP, C. J., KLOEPPER, J. W., LIFSHITZ, R., DION, P., and ANTOUN, H. 1991. Frequent occurrence of the ability to utilize octopine in rhizobacteria. Can. J. Microbiol. 37: 158–164.

Over 200 strains of root-colonizing bacteria were tested for opine catabolism. Of these, 12% utilized octopine, 9% catabolized octopinic acid, and <1% used succinamopine. None grew on mannopine or nopaline. Most of the octopine and octopinic acid utilizers were pseudomonads. Only one Gram-positive bacterium used succinamopine. No strains of *Serratia*, *Enterobacter*, *Aeromonas*, or *Bacillus* catabolized an opine. All bacteria that were isolated from roots of arctic plants and selected for dinitrogen fixation utilized octopine after enrichment cycles with malate. Malate and glucose were compared for their ability to amplify, when used as selective substrates, the fraction of opine utilizers initially present in a plant sample. With this approach, <5% of 401 rhizobacteria tested utilized octopine, octopinic acid, or both opines. The opine-catabolizing strains belonged to the families *Rhizobiaceae* and *Pseudomonadaceae* and to the genera *Alcaligenes* and *Flavobacterium*. The relative effect of malate and glucose in the enrichment for opine utilizers varied according to the origin of the sample. In this study, octopine and to a lesser extent octopinic acid were recognized as substrates that were more commonly utilized by rhizobacteria than other opines. These results demonstrate that opine catabolism is not restricted to the genus *Agrobacterium*.

Key words: octopine, rhizobacteria, *Pseudomonadaceae*, *Alcaligenes*, *Flavobacterium*.

BEAUCHAMP, C. J., KLOEPPER, J. W., LIFSHITZ, R., DION, P., et ANTOUN, H. 1991. Frequent occurrence of the ability to utilize octopine in rhizobacteria. Can. J. Microbiol. 37: 158–164.

Au-delà de 200 souches de bactéries colonisatrices de racines ont été testées pour leur aptitude à cataboliser des opines. D'entre elles, 12% ont utilisé l'octopine, 9% l'acide octopinique et <1% la succinamopine; aucune croissance n'a été obtenue avec la mannopine ou la nopaline. La majorité des utilisateurs d'octopine et d'acide octopinique ont été des pseudomonades. Une seule bactérie Gram positif a utilisé la succinamopine. Des diverses souches de *Serratia*, *Enterobacter*, *Aeromonas* ou *Bacillus*, aucune n'a catabolisé une opine. Toutes les bactéries isolées des racines de plantes arctiques et sélectionnées pour leur abilité à fixer l'azote atmosphérique ont utilisé de l'octopine, après des cycles d'enrichissement au malate. Le malate et le glucose ont été utilisés comme substrats sélectifs et comparés pour leur aptitude à amplifier les utilisateurs d'opines initialement présents dans un échantillon végétal. Dans cette approache, <5% des 401 rhizobactéries testées ont utilisé l'octopine, l'acide octopinique ou les deux opines. Les souches qui ont catabolisé les opines appartenaient aux familles *Rhizobiaceae*, *Pseudomonadaceae* et aux genres *Alcaligenes* et *Flavobacterium*. Les effets relatifs du malate et du glucose sur le plan de l'enrichissement pour les utilisateurs d'opines ont varié selon l'origine des échantillons. Dans cette étude, l'octopine et à un moindre degré l'acide octopinique ont été reconnus comme les substrats les plus communément utilisés par les rhizobactéries. Ces résultats montrent que le catabolisme des opines n'est pas restreint au genre *Agrobacterium*.

Mots-clés : octopine, rhizobactéries, *Pseudomonadaceae*, *Alcaligenes*, *Flavobacterium*.

[Traduit par la rédaction]

Introduction

The sloughed cells, mucilage, secretions, and exudates of plant roots create within the soil a narrow zone, the rhizosphere, where the growth and development of microorganisms occur (Rovira and Davey 1974). Rhizosphere bacteria belong to various genera (Gaskin *et al.* 1985) and include bacteria adapted for root colonization, termed rhizobacteria (Kloepper *et al.* 1988; Schippers 1988). Most rhizosphere bacteria can survive in the soil as saprophytes or dormant propagules until the environmental conditions change.

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* are two soil-inhabiting bacteria that actively colonize the rhizosphere of plants (Bouzar and Moore 1987; Nesme *et al.* 1987). It is only after a wound has occurred on a plant that they express their pathogenic potential. The anomalous growth of the

[1]Author to whom all correspondence should be addressed.

[2]Present address: Department of Plant Pathology, Alabama Agricultural Experiment Station, Auburn University, Auburn, Alabama 36849, U.S.A.

Printed in Canada / Imprimé au Canada

plant at the infection site is due to the introduction of part of the Ti or Ri bacterial plasmid into plant nuclear DNA, determining the production of plant hormones and opines (Chilton *et al.* 1977; Akiyoshi *et al.* 1983). Opines are subdivided into the imino diacids, mannityl opines, and phosphorylated sugars families (Petit *et al.* 1983; Chilton *et al.* 1984). Among the imino diacid opines, octopine and octopinic acid are reductive condensation products of pyruvate and arginine, and of pyruvate and ornithine, respectively. Similarly, nopaline and succinamopine are derived from the condensation of 2-ketoglutarate and arginine or asparagine, respectively. Among the mannityl opines, mannopine is the conjugate of mannose with glutamine.

The opines provide agrobacteria with a source of carbon and nitrogen (Guyon *et al.* 1980) and induce the conjugative transfer of Ti or Ri plasmids (Petit *et al.* 1978; Ellis *et al.* 1982). Thereafter, *Agrobacterium* strains can acquire a competitive advantage over the other microorganisms (Tempé and Petit 1983), which presumably are unable to utilize opines. However, the question of the selectivity of opines as growth substrates has been reviewed in previous studies documenting opine utilization by soil microorganisms, such as pseudomonads (Beaulieu *et al.* 1983; Rossignol and Dion 1985), rhizobia (Kondorosi *et al.* 1983), coryneform bacteria (Tremblay *et al.* 1987), and fungi (Beauchamp *et al.* 1990). This diversity of opine-utilizing microorganisms contrasts with the specialized sites at which opines are produced in nature.

Previous studies on opine catabolism have usually involved bacteria selected on the basis of their ability to utilize opines. However, no comprehensive analysis of root-bacteria capacity to utilize opines has been reported. In the present study, a collection of rhizobacteria that were known plant-root colonizers and that were isolated on opine-free medium was screened for opine utilization. The results suggested that enrichment cycles amplify the initial fraction of opine-utilizing bacteria from a plant sample. This possibility was further examined using malate and glucose and three types of plants to determine the effect of the enrichment compound and of the host. The diversity among octopine utilizers was observed to be greater than initially suspected and the host plant appeared to determine the nature of its opine-utilizing rhizospheric population.

A preliminary report on part of these results has been published (Beauchamp *et al.* 1988).

Materials and methods

Chemicals

Mannopine and the mixture of D,L- and L,L-succinamopine (1:1) were a gift from Dr. W. S. Chilton (Raleigh, NC). The other test substrates were from Sigma Chemical Co. (St. Louis, MO).

Bacterial strains

Rhizobacterial strains were obtained from the collection of Allelix Crop Technologies Inc. (Mississauga, Ont., Canada). In general, these bacteria were isolated from plant roots collected from various Canadian locations, and a few were isolated from soil or water. Sixteen of the Gram-negative rhizobacteria included in the rhizobacterial collection, comprising *Pseudomonas fluorescens*, *Pseudomonas putida*, and unidentified strains, had been isolated from the Northwest Territories (Lifshitz *et al.* 1986) or Labrador and had the ability to fix dinitrogen. Moreover, three Tn*5* mutants of *P. putida* defective in dinitrogen-fixation ability were also included in this study (Lifshitz *et al.* 1987). All strains of the rhizobacterial collection colonized roots of canola (*Brassica campestris*) or soybean (*Glycine max*) at population densities of log 3 colony-forming units (cfu) per gam root or greater (Kloepper *et al.* 1988; Polonenko *et al.* 1987). Isolates of the enrichment culture collection were obtained as described below.

Culture media

The capacity for opine utilization was evaluated using AT salts basal medium (AT-B) of Guyon *et al.* (1980), AT-B supplemented with glucose (2 $g \cdot L^{-1}$) and ammonia sulfate (1 $g \cdot L^{-1}$) (AT-CN), or AT-B supplemented with a test substrate as previously described (Beauchamp *et al.* 1990). The test opines were added as the sole carbon and nitrogen source at a final concentration of 0.8 $g \cdot L^{-1}$, except for a mixture of D,L-succinamopine and L,L-succinamopine (1:1), which was added at 1.6 $g \cdot L^{-1}$. The pH of the media was adjusted to 7.0. When required, AT-CN medium was solidified by adding 1.5% Bacto agar (Difco Laboratories, Detroit, MI).

The acetylene-reduction activity was determined using a nitrogen-free medium containing the following per litre of distilled water: malic acid, 5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 2 mg; $MnSO_4$, 2.4 mg; H_3BO_3, 2.8 mg; vitamins as described by Beauchamp *et al.* (1990); and Noble agar, 1.75 g (Difco).

For the enrichment cycles, the selective media were similar to the nitrogen-free medium, except that 5 $g \cdot L^{-1}$ of L-malic acid or glucose was used as the carbon source in the presence or absence of 1 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$. The pH was adjusted to 7.0 with KOH (6 M).

Determination of the capacity for opine utilization

Bacteria were grown for 2 to 3 days at room temperature on AT-CN solid medium. The inoculum was prepared by suspending the bacteria in 5 mL of AT-B medium to give an optical density of 0.1–0.2 at 660 nm, and by further diluting this suspension 10-fold in AT-B medium. A portion (0.1 mL) of inoculum was transferred to 1 mL of AT-B, AT-CN, or AT-B supplemented with a test substrate. Cultures were incubated at room temperature and growth was evaluated visually for up to 28 days, with shaking at 100 rpm. The results in AT-B and AT-CN represented negative and positive responses for all experiments, respectively. Experiments were performed three times.

For strains catabolizing octopinic acid or octopine, the utilization of these opines was confirmed by high-voltage paper electrophoresis followed by paper detection with the ninhydrin or phenanthrenequinone reagent (Beauchamp *et al.* 1990).

Determination of acetylene-reduction activity

Bacteria were tested for acetylene-reduction activity (ARA) at 13 and 20 ± 2°C. After 7 days of growth in nitrogen-free medium, ARA was measured as follows. The flasks were plugged with Subba seals (William Freeman Company Ltd., Barnsley, England) and 10% (v/v) of the air was replaced with acetylene. The cultures were incubated for 30 min, and ethylene concentration was assayed by injecting a 1-mL sample in a gas chromatograph (model sigma 3B; Perkin-Elmer, Montréal, Que., Canada) equipped with a flame ionization detector and a 1-m Porapak R (100-120 mesh) column. The detector, injection port, and oven temperatures were set at 125, 55, and 45°C, respectively. Only qualitative information was recorded (i.e., absence or presence of ARA). Each test was performed three times.

Isolation of bacteria through enrichment cycles

Bacteria were isolated during the fall of 1986 from the roots of field-grown corn (*Zea mays* L.), potato (*Solanum tuberosum* L.), and spring wheat (*Triticum aestivum* L.). Roots were processed in the laboratory within 4 h after harvest. The roots were washed under tap water and the remaining soil was gently removed with distilled water. Washed root pieces (0.1 g), or 0.1 mL of a suspension containing 1 g of root pieees homogenized in 99 mL of 0.1 M $MgSO_4$, were added to 20 mL of a selective enrichment medium in 50-mL flasks.

In addition, 0.1 mL of the homogenized root piece suspensions was plated on the enrichment media solidified with 1.5% Noble agar (Difco) and on tryptic soy agar (TSA, Difco) (referred to as cycle 0). After 1 week of incubation at room temperature 150 μL from each flask was used to inoculate fresh medium for the next enrichment cycle. After enrichment cycles 2, 6, and 10, colonies were isolated on TSA. Colonies representative of morphological types present on the plates were selected and purified on TSA. Single colonies were then evaluated for their ability to utilize octopine and octopinic acid as described above. Opine-utilizing isolates were also tested for ARA.

TABLE 1. Catabolism of crown-gall opines by strains from the rhizobacterial collection after 28 days of growth

Bacteria and strain No.	No. of strains tested	Dinitrogen-fixation capacity[a]	Opine utilized[b]		
			Oct	Oca	Sap
Gram negative					
P. fluorescens	63				
G12.117, G25.54		−	+	+	−
G11.46		−	+	−	−
GR20.3		+	+	+	−
P. putida	61				
29.420, G2.10, G3.9, 61.9, G25.34		−	+	+	−
G11.57		−	+	−	−
GR7.4R2, GR12.2, GR12.7, GR17.4					
GR19.1, GR20.5		+	+	+	−
GR2.11, GR8.17, GR25.5, GR25.6		+	+	−	−
Serratia liquefaciens	28				
Unidentified	21				
86.65		−	+	+	−
GR3.5, GR8.8, GR25.97		+	+	+	−
L16.33, L41.3		+	+	−	−
Enterobacter sp.	3				
Aeromonas hydrophila	2				
Total	176		26	18	0
Gram positive					
Unidentified	25				
86.139			−	−	+
Bacillus sp.	7				
Total	32		0	0	1

[a]All positive strains were of arctic origin and all GR strains were isolated from enrichment cycles on malate.
[b]Oct, octopine; Oca, octopinic acid; Sap, D,L- and L,L-succinamopine. All strains were negative on nopaline and mannopine.

Diagnostic tests of the opine-utilizing isolates

For morphological and biochemical tests, the inoculum was prepared by growing bacteria on nutrient agar or nutrient broth (Difco) supplemented with 5 $g \cdot L^{-1}$ of yeast extract (NAYE or NBYE) at 25 ± 2°C for 24–48 h.

The cell size and the number and arrangement of flagella was determined by electron microscopy using negatively stained preparations (Tremblay *et al.* 1987). The Gram reaction was determined on 24-h-old cultures by using the KOH technique (Suslow *et al.* 1982). The results of the glucose oxidation–fermentation test (MacFaddin 1980) were used to distinguish between aerobic and facultative anaerobic bacteria. The presence of catalase and oxidase enzymes was evaluated by using 30% H_2O_2 and *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride reagents, respectively (MacFaddin 1980). To assess motility, the strains were inoculated at the center of a Petri dish containing 20 mL of NAYE semisolid medium containing 0.3% agar. Bacteria were considered motile when the colony diameter was >5 mm after 2 days of incubation. Other tests performed were the hydrolysis of aesculine, Tween-80, starch, urea (MacFaddin 1980), and gelatin (Tremblay *et al.* 1987); the production of acetoin, arginine dihydrolase, and indole from tryptophane and peptone; the production of 3-ketolactose, a pellicle in ferric ammonium citrate broth (Moore *et al.* 1988), levan (Stolp and Gadkari 1981), pyocyanine, and pyoverdine (Bergan 1981); methyl red, denitrification (MacFaddin 1980); and growth at 4 and 41°C (Stolp and Gadkari 1981), in the presence of 5% NaCl, and on *Agrobacterium* selective media 1A and 2E of Brisbane and Kerr and on the adonitol medium of Roy and Sasser (Moore *et al.* 1988). The results from all these tests were determined 7–8 days after inoculation, except for the urease test, where the results were taken after 2 days of growth. All test were performed at 25 ± 2°C, unless otherwise specified.

The oncogenicity tests were performed on carrot slice (*Daucus carota*), tobacco (*Nicotinia tobaccum*), and tomato (*Lycopersicon esculentum*) plants (Moore *et al.* 1988), whereas the nodulation tests were performed on alfalfa (*Medicago sativa*), faba bean (*Vicia faba*), pea (*Pisum sativum*), and soya (*Glycine max*) (Vincent 1970). The presence of tumors and nodules was evaluated 1 month after the inoculation.

Results

Evaluation of rhizobacteria for opine utilization and ARA

Over 200 rhizobacteria were tested for opine utilization (Table 1). None of them was able to use nopaline or mannopine. The strains varied in their capacity to utilize octopine and octopinic acid. After up to 7 days, some had completely utilized both opines, whereas others had catabolized octopine only. Many octopine-utilizing bacteria exhibited growth on octopinic acid after a period of 14–28 days. On average, 15% of the Gram-negative bacteria used octopine within 7 days. All of the identified octopine utilizers belonged to the *P. putida* – *P. fluorescens* group. Only one of the rhizobacteria tested, an unidentified Gram-positive strain, utilized succinamopine (Table 1).

All of the 16 dinitrogen-fixing bacteria of arctic origin (GR and L strains; Table 1) utilized octopine. Three Tn*5* mutants of the arctic *P. putida* GR12.2 impaired in their acetylene-reduction activity utilized octopine like the wild type. None of the other rhizobacteria performed dinitrogen fixation.

TABLE 2. Isolation of opine-utilizing bacteria through enrichment cycles

Host	Cycle of enrichment	No. of isolates tested for opine catabolism	Designation of opine utilizer	Isolates identified	Selective medium used for recovery of opine utilizer	Opine utilized	
						Oct	Oca
Corn	0	18	CJ32	Fluorescent pseudomonad	TSA	+	
	2	29	CJ197	*Alcaligenes* sp.	M		+
	6	38	—				
	10	40	CJ369	*Alcaligenes* sp.	MN		+
Total		125	3				
Potato	0	31	—				
	2	38	CJ66	Nonfluorescent pseudomonad	GN	+	+
	6	34	CJ251	Nonfluorescent pseudomonad	GN	+	+
			CJ253	Nonfluorescent pseudomonad	MN	+	+
			CJ286	*Rhizobiaceae*	GN	+	+
	10	39	CJ376		G		+
			CJ425		M		+
			CJ439		M		+
			CJ451		GN	+	+
			CJ452	*Alcaligenes* sp.	GN	+	+
Total		142	9				
Wheat	0	27	CJ38	*Flavobacterium* sp.	G		+
	2	40	CJ221	*Rhizobizceae*	M	+	+
	6	31	CJ287	*Rhizobiaceae*	M	+	+
			CJ310	Fluorescent pseudomonad	M	+	
			CJ321	*Rhizobiaceae*	M	+	+
	10	36	CJ460		M	+	+
			CJ474		M		+
Total		134	7				

NOTE: Oct, octopinic; Oca, octopine acid; TSA, triptic soy agar; M, malic acid; MN, malic acid + nitrogen; G, glucose; GN, glucose + nitrogen.

Isolation of bacteria through enrichment cycles on malate or glucose

Most of the dinitrogen-fixing bacteria included in the rhizobacterial collection had been obtained from plant samples and through enrichment cycles in a medium containing malate as the carbon source, and no added nitrogen. Because all of these arctic bacteria utilized octopine, it seemed possible that an unexpected effect of the enrichment cycles had been to select for octopine utilizers. To test this hypothesis, a new series of isolations by enrichment cycles was performed, this time using inocula collected in the Québec, Que., area.

A total of 401 bacteria isolated from cycles 0, 2, 6, and 10 on TSA and able to grow on AT-CN medium were evaluated for opine catabolism (Table 2). Cycle 0 yielded the greatest diversity in colony morphology. This variability decreased over successive enrichment cycles. On TSA, the typical colony morphologies were whitish to creamy, with an entire edge, while other colonies were mucoid. In general, from cycle 6 on, only the opine-utilizing isolates with distinct colony morphological types were identified using morphological and biochemical tests.

Three opine-utilizing isolates were obtained from corn roots (Table 2). Nine opine utilizers were isolated from potato roots; most of these were selected on a medium with glucose and nitrogen. Finally, seven opine-utilizing isolates were recovered from wheat roots; all but one were selected on medium with malate. The opine-utilizing isolates which were subjected to determinative tests were classified in five different groups of Gram-negative bacteria (Tables 2 and 3). None of the opine-utilizing isolates exhibited ARA.

Discussion

Previous studies on opine catabolism by microorganisms have usually been initiated by selecting bacteria on the basis of their ability to utilize opines or as members of the genus *Agrobacterium*. This study presents a systematic evaluation of opine-catabolism capacity by rhizobacterial strains. These strains were isolated on opine-free media for their ability to colonize roots and favour plant growth. The ability to utilize octopine within 7 days was of particularly common occurrence among the fluorescent pseudomonads tested. Given the dilute inoculum that was used, such a rapid response suggests that growth on this substrate is a character of the isolate as recovered from nature, rather than the product of spontaneous mutation and selection on octopine. However, spontaneous mutation probably accounts for the delayed growth on octopinic acid that is observed for many strains. The capacity of octopine-utilizing pseudomonads to mutate spontaneously to octopinic acid catabolism has been

TABLE 3. Morphological and physiological characteristics of opine-utilizing bacteria isolated through enrichment cycles

			Pseudomonadaceae		
	Alcaligenes	*Flavobacterium*	Fluorescent	Nonfluorescent	*Rhizobiaceae*
Morphology	Rod	Rod	Rod	Rod	Rod
Cell size (μm)					
Width	0.7–0.8	0.3–0.4	0.7–0.8	0.6–0.9	0.6–1.0
Length	2.0–2.5	2.0–3.0	2.0–2.4	2.0–3.0	1.0–3.0
No. of flagella	1 to >1	Absent	1 to >1	>1	1 to >1
Flagellar arrangement	Peritrichous		Polar	Polarperitrichous	Peritrichous
Gram (KOH)	−	−	−	−	−
Glucose oxidation	+	−	+	+	+
Glucose fermentation	−	−	−	−	−
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	−	+	+	+
Hydrolysis of:					
aesculine	−	−	−	−	+
gelatine	−	−	−	−	−
lipase (Tween-80)	−	−	−	−(1/3)	−
starch	−(1/3)	−	−	−	−
urea	−	+	+	+	+
Production of:					
acetoin	−	−	−	−	−
arginine dihydrolase	−	−	+	+	+
indole	−	−	−	−	−
3-ketolactose	−	−	−	−	−
levan	−	−	−	+(1/3)	+
methyl red	−	−	−	−	−
NO_2	+(1/3)	+	+	−(1/3)	+
N_2	−	−	+	−	+
pellicle	−	−	−	−	−
pyocyanin	−	−	−	−	−
pyoverdin	−	−	+	−	−
Growth at:					
4°C	+	+	+	+	+
41°C	+	−	−	+(1/3)	±(2/3)
Growth on:					
adonitol	−	−	−	−	+
arabitol (A1 medium)	−	−	−	−	+(1/3)
erythritol (E2 medium)	−	−	−	+(1/3)	+(2/3)
NaCl 5%	+	−	−	+(1/3)	−
Tumor induction	−	−	−	−	−
Nodule induction	−	−	−	−	−

NOTE: Total number of strains tested for *Alcaligenes*, 3; *Flavobacterium*, 1; fluorescent *Pseudomonadaceae*, 2; nonfluorescent *Pseudomonadaceae*, 3; *Rhizobiaceae*, 4.

reported before (Rossignol and Dion 1985). Succinamopine utilization was not observed among the Gram-negative members of the collection but was noted in one Gram-positive rod. Succinamopine catabolism by coryneform bacteria has been reported previously (Tremblay *et al.* 1987). None of the bacteria evaluated catabolized nopaline or mannopine.

The rhizobacterial collection included a subset of 16 arctic bacteria, which shared the capacity to fix dinitrogen (Liftshitz *et al.* 1986). All 16 strains grew rapidly on octopine but not on octopinic acid. Conceivably, the nitrogen-fixation and octopine-utilization activities could require a common metabolic step, which would often be limiting for octopine degradation. This common step would still operate in the Tn*5*, mutants impaired in nitrogen fixation that were studied here, since they still utilized octopine. The observed structural similarity between rhizobactin, a siderophore from *Rhizobium meliloti*, and octopine (Smith and Neilands 1987) may be indicative of the nature of the putative relationship between nitrogen fixation and opine catabolism. Opine may regulate iron assimilation, an element required in large quantities for nitrogen fixation, or else may be involved in modifying the NAD^+/NADH ratio under low O_2 tension. Another possible explanation for the universal occurrence of the capacity for octopine catabolism is that this capacity correlates not with nitrogen fixation but with the arctic origin of the isolates. Finally, the protocol used for recovery of these bacteria, involving enrichment cycles on malate, and in the absence of added fixed nitrogen, could have coselected for nitrogen-fixing and octopine-utilizing bacteria.

The latter possibility was evaluated, this time using plant samples collected from a temperate region and an incubation temperature of 20°C, instead of 14°C as in the original study

(Lifshitz *et al.* 1986). Of the strains isolated in this manner, <5% utilized octopine or octopinic acid. This proportion is lower than the percentage of octopine or octopinic acid utilizers in the rhizobacterial collection, even when the bacteria of arctic origin are not considered. Thus, it appears that neither enrichment on malate *per se* nor modifications to the enrichment medium by adding fixed nitrogen or changing the carbon source amplifies the initial fraction of opine utilizers within a plant-root sample. None of the opine-utilizing bacteria showed acetylene-reduction activity, confirming that the conditions that led previously to the recovery of the opine utilizers of arctic origin were not reproduced here.

Most of the opine-utilizing potato isolates were obtained on enrichment media containing glucose and ammonium sulfate. By contrast, the wheat isolates were preferentially recovered on malate, and in the absence of fixed nitrogen. Also, most of the *Pseudomonadaceae* were isolated from potato, whereas most of the *Rhizobiaceae* were obtained from wheat. Even though only three bacteria were isolated from corn, two of them belonged to the genus *Alcaligenes*. All opine-utilizing bacteria under investigation in this study belong to genera recognized as rhizospheric colonizers (Gaskins *et al.* 1985; Kloepper *et al.* 1988; Schippers 1988). This suggests that different opine-utilizing bacteria are associated with different host plants. Plants have a selective effect on the microbial population associated with their rhizosphere (Miller *et al.* 1989), this effect being determined by root exudates (Atkinson *et al.* 1975; Rovira and Davey 1974).

The present study identifies the genera *Alcaligenes* and *Flavobacterium* as two new genera comprising opine-utilizing representatives. The pattern of opine catabolism differed among the genera under investigation. Most of the representatives of the *Alcaligenes* and *Flavobacterium* genera catabolized only octopinic acid; the fluorescent pseudomonads used octopine only, whereas the nonfluorescent pseudomonads and the *Rhizobiaceae* grew on octopine and octopinic acid. The relatively frequent occurrence of opine catabolism in a diverse array of bacterial types must be reconciled with the fact that opines are generally specific to plant tissues genetically transformed by *Agrobacterium*. This catabolic ability is in part a reflection of the nutritional versatility of the pseudomonads. On the other hand, members of the *Alcaligenes* and *Flavobacterium* genera do not generally show such a versatility (Kreig and Holt 1984; C. J. Beauchamp. 1989. Ph.D. thesis, Université Laval, Ste-Foy).

In the present study, octopine and octopinic acid were identified as two opines more frequently utilized by Gram-negative bacteria than were nopaline, succinamopine, and mannopine. Bacteria capable of octopine or octopinic acid catabolism were found to be distributed among at least five genera of Gram-negative bacteria. While some of the isolates were identified as members of the family *Rhizobiaceae*, none induced tumors on any of the host plants tested. The isolation of opine-utilizing, but avirulent agrobacteria has also been described in other studies (Beaulieu *et al.* 1983; Tremblay *et al.* 1987). Thus, the results reported here reveal a distinctive feature of the catabolism of octopine and octopinic acid by bacteria, as these opines appear of little selective value for pathogenic agrobacteria. The selectivity of other opines such as mannopine, nopaline, and succinamopine may differ from that of octopine and octopinic acid. In keeping with the general occurrence and diverse characteristics of octopine and octopinic acid utilizers, it was also shown here that the nature of these bacteria varies with the host plant and is not strictly related to the crown-gall system.

Acknowledgements

The authors thank M. Laliberté for her technical assistance during the visit of C. J. Beauchamp to Allelix Crop Technologies, Mississauga, Ont. This work was supported by grants from the Fonds pour la formation de chercheurs et l'aide à la recherche and from the Ministère de l'enseignement supérieur et de la science du Québec–action structurante. C. J. Beauchamp acknowledges the receipt of a postgraduate scholarship from the National Sciences and Engineering Research Council of Canada.

AKIYOSHI, D. E., MORRIS, R. O., HINZ, R., MISCHKE, B. S., KOSUGE, T., GARFINKEL, D. J., GORDON, M. P., and NESTER, E. W. 1983. Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proc. Natl. Acad. Sci. U.S.A. **80**: 407–411.

ATKINSON, T. G., NEAL, J. L., and LARSON, R. I. 1975. Genetic control of the rhizosphere microflora of wheat. *In* Biology and control of soil-borne plant pathogens. *Edited by* G. W. Bruehl. American Phytopathological Society Press, St. Paul, MN. pp. 116–122.

BEAUCHAMP, C. J., DION, P., KLOEPPER, J. W., LIFSHITZ, R., LALIBERTÉ, M., and ANTOUN, H. 1988. Utilization of crown-gall opines by dinitrogen-fixing pseudomonads isolated from the Canadian high arctic. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. de Bruijn, and W. E. Newton. Gustav Fischer, New York. p. 649.

BEAUCHAMP, C. J., CHILTON, W. S., DION, P., and ANTOUN, H. 1990. Fungal catabolism of crown-gall opines. Appl. Environ. Microbiol. **56**: 150–155.

BEAULIEU, C., COULOMBE, L. J., GRANGER, R. L., MIKI, B., BEAUCHAMP, C., ROSSIGNOL, G., and DION, P. 1983. Characterization of opine-utilizing bacteria isolated from Québec. Phytoprotection, **64**: 61–68.

BERGAN, T. 1981. Human- and animal-pathogenic members of the genus *Pseudomonas*. *In* The prokaryotes. A handbook on habitats, isolation, and identification of bacteria. Vol. 1. *Edited by* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel. Springer-Verlag, New York. pp. 666–700.

BOUZAR, H., and MOORE, L. W. 1987. Isolation of different *Agrobacterium* biovars from a natural oak savanna and tallgrass prairie. Appl. Environ. Microbiol. **53**: 717–721.

CHILTON, M.-D., DRUMMOND, M. H., MERLO, D. J., SCIAKY, D., MONTOYA, A. L., GORDON, M. P., and NESTER, E. W. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown-gall tumorigenesis. Cell, **11**: 263–271.

CHILTON, W. S., TEMPÉ, J., MATZKE, M., and CHILTON, M.-D. 1984. Succinamopine: a new crown gall opine. J. Bacteriol. **157**: 357–362.

ELLIS, J. G., KERR, A., and TEMPÉ, J. 1982. Conjugal transfer of nopaline and agropine Ti plasmids—the role of agrocinopines. Mol. Gen. Genet. **186**: 269–274.

GASKINS, M. H., ALBRECHT, S. K., and HUBBELL, D. H. 1985. Rhizosphere bacteria and their use to increase plant productivity: a review. Agric. Ecosyst. Environ. **12**: 99–116.

GUYON, P., CHILTON, M.-D., PETIT, A. and TEMPÉ, J. 1980. Agropine in "null-type" crown gall tumors: evidence for generality of the opine concept. Proc. Natl. Acad. Sci. U.S.A. **77**: 2693–2697.

KLOEPPER, J. W., HUME, D. J., SCHER, F. M., SINGLETON, C., TIPPING, B., LALIBERTÉ, M., FRAULEY, K., KUTCHAW, T., SIMONSON, C., LIFSHITZ, R., ZALESKA, I., and LEE, L. 1988. Plant growth-promoting rhizobacteria on canola (rapeseed). Plant Dis. **72**: 42–46.

KONDOROSI, A., KONDOROSI, E., BANFALVI, Z., BROUGHTON, W. J.,

PANKHURST, C. E., RANDHAWA, G. S., WONG, C.-H., and SCHELL, J. 1983. Analysis of symbiotic nitrogen fixation genes carried by the *Rhizobium melioti* mega-plasmid. *In* Molecular genetics of the bacteria–plant interaction. *Edited by* A. Pühler. Springer-Verlag, New York. pp. 55–63.

KRIEG, N. R., and HOLT, J. G. (*Editors*). 1984. Bergey's manual of systematic bacteriology. Vol. 1. Williams and Wilkins Co., Baltimore.

LIFSHITZ, R., KLOEPPER, J. W., SCHER, F. M., TIPPING, E. M., and LALIBERTÉ, M. 1986. Nitrogen-fixing pseudomonads isolated from roots of plants grown in the Canadian High Arctic. Appl. Environ. Microbiol. **51**: 251–255.

LIFSHITZ, R., KLOEPPER, J. W., KOZLOWSKI, M., SIMONSON, C., CARLSON, J., TIPPING, E. M., and ZALESKA, I. 1987. Growth promotion of canola (rapeseed) seedlings by strain of *Pseudomonas putida* under gnotobiotic conditions. Can. J. Microbiol. **33**: 390–395.

LIPPINCOTT, J. A., LIPPINCOTT, B. B., and STARR, M. P. 1981. The genus *Agrobacterium*. *In* The prokaryotes. A handbook on habitats, isolation, and identification of bacteria. Vol. 1. *Edited by* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel. Springer-Verlag, New York. pp. 842–855.

MACFADDIN, J. F. 1980. Biochemicals tests for identification of medical bacteria. Williams & Wilkins Co., Baltimore.

MILLER, H. J., HENKEN, G., and VAN VEEN, J. A. 1989. Variation and composition of bacterial populations in the rhizospheres of maize, wheat, and grass cultivars. Can. J. Microbiol. **35**: 656–660.

MOORE, L. W., KADO, C. I., and BOUZAR, H. 1988. *Agrobacterium*. *In* Plant pathogenic bacteria. *Edited by* N. W. Schaad. American Phytopathological Society Press, St. Paul, MN. pp. 16–36.

NESME, X., MICHEL, M.-F., and DIGAT, B. 1987. Population heterogeneity of *Agrobacterium tumefaciens* in galls of *Populus* L. from a single nursery. Appl. Environ. Microbiol. **53**: 655–659.

PETIT, A., TEMPÉ, J., KERR, A., HOLSTERS, M., VAN MONTAGU, M., and SCHELL, J. 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti-plasmids. Nature (London), **271**: 570–571.

PETIT, A., DAVID, C., DAHL, G. A., ELLIS, J. G., GUYON, P., CASSE-DELBART, F., and TEMPÉ, J. 1983. Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol. Gen. Genet. **190**: 204–214.

POLONENKO, D. R., SCHER, F. M., KLOEPPER, J. W., SINGLETON, C. A., LALIBERTÉ, M., and ZALESKA, I. 1987. Effects of root colonizing bacteria on nodulation of soybean roots by *Bradyrhizobium japonicum*. Can. J. Microbiol. **33**: 498–503.

ROSSIGNOL, G., and DION, P. 1985. Octopine, nopaline, and octopinic acid utilization in *Pseudomonas*. Can. J. Microbiol. **31**: 68–74.

ROVIRA, A. D., and DAVEY, C. B. 1974. Biology of the rhizosphere. *In* The plant root and its environment. *Edited by* E. W. Carson. University Press of Virginia, Virginia. pp. 153–204.

SCHIPPERS, B. 1988. Biological control of pathogens with rhizobacteria. Philos. Trans. R. Soc. London, Ser. B, **318**: 283–293.

SMITH, M. J., and NEILANDS, J. B. 1987. Rhizobactin, a structurally novel siderophore biochemically related to the opines. *In* Molecular strategies for crop protection. *Edited by* C. J. Arntzen and C. Ryan. A. R. Liss, Inc., New York. pp. 157–168.

STOLP, H., and GADKARI, D. 1981. Nonpathogenic members of the genus *Pseudomonas*. *In* The prokaryotes. A handbook of habitats, isolation, and identification of bacteria. Vol. 1. *Edited by* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel. Springer-Verlag, New York. pp. 719–741.

SUSLOW, T. V., SCHROTH, M. N., and ISAKA, M. 1982. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology, **72**: 917–918.

TEMPÉ, J., and PETIT, A. 1983. La piste des opines. *In* Molecular genetics of the bacteria–plant interaction. *Edited by* A. Pühler. Springer-Verlag, New York. pp. 14–32.

TREMBLAY, G., GAGLIARDO, R., CHILTON, W. S., and DION, P. 1987. Diversity among opine-utilizing bacteria: identification of coryneform isolates. Appl. Environ. Microbiol. **53**: 1519–1524.

VINCENT, J. M. 1970. A manual for the practical study of the root nodule bacteria. IBP Handbook No. 15. Blackwell Scientific Publications, Oxford.