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Physiological characterization of opine-utilizing rhizobacteria for traits related to plant growth-promoting activity

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Abstract

Thirty-two strains of opine-utilizing rhizobacteria were evaluated for physiological traits which have been related to plant growth-promoting activity. Tests included antibiosis against two bacterial and eight fungal pathogens of potato (*Solanum tuberosum* L.), production of hydrogen cyanide and fluorescent pigment production. On average, 71 and 12% of the bacteria inhibited the growth of *Erwinia carotovora* subsp. *carotovora* and *Agrobacterium tumefaciens*, respectively. The growth of *Botrytis* sp. was inhibited by 62% of the bacteria, and half of these produced an inhibition zone of more than 7 mm in diameter. *Fusarium solani*, *Colletotrichum coccodes*, *Phoma exigua*, *Verticillium dahliae*, *F. oxysporum*, *V. albo-atrum* and *F. sambucinum* were antagonized by 43, 34, 31, 25, 19, 18, and 12% of the bacteria, respectively. Only four strains produce hydrogen cyanide. The inhibition of a plant pathogen was not correlated to the production of fluorescent pigment. No strain produced a hypersensitive reaction whereas only three strains induced soft-rot and two produced polygalacturonase. Some opine-utilizing rhizobacteria were strong inhibitors of all plant pathogens, while most were active against specific plant pathogens.

Introduction

Rhizobacteria represent the subclass of total rhizosphere bacteria capable of colonizing roots (Schroth and Hancock, 1982). Root colonization is defined as a process whereby bacteria survive inoculation onto seeds, multiply in the spermosphere in response to seed exudates, transfer to the developing root system, and multiply on roots (Kloepper *et al.*, 1989). The effects of rhizobacteria on the inoculated host plant may be beneficial, deleterious or neutral (Schippers *et al.*, 1987). Beneficial rhizobacteria are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978).

Growth promotion by PGPR may occur by one of two general mechanisms; direct or indirect growth promotion (Kloepper *et al.*, 1988). Direct growth promotion occurs when PGPR metabolites stimulate plant growth independent of native root zone microflora (Lifshitz *et al.*, 1987). In contrast, indirect growth promotion occurs when PGPR displace or antagonize native deleterious microflora. Indirect growth promotion is involved with most published accounts of PGPR (Kloepper and Schroth, 1978). Some of

the displaced indigenous root zone microorganisms are deleterious to plants (Schippers, 1988), including deleterious rhizosphere microorganisms (DRM). DRM are saprophytic pathogens which reduce root growth of plants (Alström, 1989; Bakker and Schippers, 1987; Suslow, 1982). Growth promotion by indirect-acting PGPR is associated with antibiosis *in vitro* to indigenous microflora including DRM (Suslow, 1982) and plant pathogens (Kloepper and Schroth, 1978). Hence, indirect growth promotion is, in effect, biological control. In addition to providing control of DRM, some PGPR and some rhizobacteria have been reported to be biological control agents of parasitic plant pathogens (Kloepper, 1990; Weller, 1988).

Both growth promotion and biological control by PGPR are predicated upon root colonization of the host plant. Mechanisms by which bacteria colonize roots are not clearly known. However, some factors which have been implicated with specific PGPR include generation time (Scher *et al.*, 1988), motility (de Weger *et al.*, 1987), chemotaxis (Scher *et al.*, 1985), and adhesion (James *et al.*, 1985). Another bacterial trait which could theoretically relate to root colonization is growth on plant-released substances responsible for nutritional selection.

Opines are one class of such plant-specific compounds (Tempé and Petit, 1983). They are produced in crown-gall tumors following infection by virulent agrobacteria. Crown-gall opines are rare compounds in nature, being mainly encountered within the plant tumor environment, and yet, the capacity to catabolize opines may represent an overall affinity for root association which could favor root colonization. Opines are selective nutrients catabolized by a restricted group of bacteria (Beauchamp *et al.*, 1990b; Beaulieu *et al.*, 1983; Bouzar *et al.*, 1987; Rossignol and Dion, 1985; Tremblay *et al.*, 1987) and fungi (Beauchamp *et al.*, 1990a). Among the non-agrobacteria reported to catabolize opine, most belong to genera recognized as root colonizers (Beauchamp *et al.*, 1990b). The utilization of opine by rhizobacteria could select for efficient catabolism of carbohydrate and amino acids, which could correlate with the ability to catabolize many organic compounds found in the rhizosphere. This trait could select for better rhizosphere competence and more efficient biocontrol when present in PGPR.

The objective of this study was to determine if opine-utilizing rhizobacteria have certain physiological traits which have been previously associated with indirect growth promotion by PGPR, as a first step toward comparing their *in vivo* efficacy with known PGPR. Accordingly, antibiosis *in vitro* against two bacterial and eight fungal pathogens of potato on a low-iron medium, production of cyanide, and fluorescent pigment production were evaluated. In addition, the strains were evaluated for physiological traits associated with pathogenesis, including hypersensitive reaction on tobacco, and potato soft rot induction, and the presence of pectolytic enzymes.

Materials and methods

Microorganisms

The opine-utilizing rhizobacteria used in this study were obtained from Allelix Crop Technologies (6850 Goreway Drive, Mississauga, Ontario, Canada). These strains were selected for their ability to colonize roots of canola (rapeseed) and soybean (Kloepper *et al.*, 1988; Lifshitz *et al.*, 1987; Polonenko *et al.*, 1987). In addition, five opine-utilizing bacteria were used; two of them were isolated from potato tubers (strains 1.9 and 4.6; Beauchamp, unpublished), and three strains were isolated from crown-gall tumors and obtained from the culture collection of Laval University (Dr P Dion, Département de phytologie, Université Laval, Ste-Foy, Québec, Canada; strains 203, 211, 212). All these strains catabolize octopine, octopinic acid, or nopaline (Beauchamp *et al.*, 1990a; Beauchamp, Ph D thesis, Université Laval, Ste-Foy, Québec, Canada, 1989).

The target organisms for antibiosis studies were pathogenic bacteria; *Agrobacterium tumefaciens* (strain B6S3; J. Tempé, Institut des Sciences Végétales, Centre National de la Recherche Scientifique, 91198 Gif sur Yvette, France) and *Erwinia carotovora* subsp. *carotovora* (strain 26; S. H. DeBoer Research Station

Agriculture Canada, Vancouver, BC, Canada), and eight pathogenic fungi of potato (*Solanum tuberosum* L.) listed in Table 2. These fungi were obtained from various sources as described previously (Beauchamp *et al.* 1990a).

Bacterial and fungal inocula

All bacteria were grown on potato dextrose agar (PDA) (Difco) for 3 days. The cells were harvested and washed twice in 0.1 *M* $MgSO_4$. The optical density was adjusted to 0.3 (A_{660}), which resulted in Log 8 cells mL^{-1}.

Fungi were grown on potato dextrose agar for 14 days at room temperature. Spores were collected by washing the agar surface with 10 mL of sterile distilled water. The spore suspensions were then adjusted to Log 6 spores mL^{-1}.

Evaluation of antagonistic activity of opine-utilizing bacteria to pathogens

Inhibition of the pathogenic bacteria and fungi was tested on Pseudomonas agar F (PAF, Difco Laboratories, Detroit, Mich., USA). A 10 μL sample of a bacterial suspension was placed in a Petri dish; four equidistant drops were used per Petri dish. Bacteria were grown at room temperature for two days, then the suspensions of pathogenic bacteria and fungi were atomized over the media. Plates were further incubated at room temperature in the dark and the inhibition

Table 1. Ability of opine-utilizing rhizobacteria to inhibit the growth of various bacterial potato pathogens

Identification	Strain number	Inhibition zone (mm)	
		Erwinia carotovora	*Agrobacterium tumefaciens*
P. fluorescens	61.9A	11.0	0.0
P. aureofaciens	211	9.7	0.0
P. putida	GR8.17	9.7	0.0
P. putida	GR17.4	9.0	0.0
P. putida	GR20.5	9.0	0.0
P. putida	G25.34	8.0	1.7
P. putida	GR25.5	8.0	0.0
P. putida	GR25.6	7.7	0.0
P. putida	GR19.1	7.0	0.0
P. putida	GR12.2	7.0	0.0
P. fluorescens	GR20.3	6.4	0.0
P. putida	GR7.4R2	6.7	0.0
P. aureofaciens	212	5.0	0.0
P. putida	G3.9	5.0	0.0
pseudomonad	1.9	3.3	0.0
pseudomonad	4.6	3.3	0.3
P. putida	203	3.0	6.3
pseudomonad	L16.33	2.0	1.7
P. fluorescens	G12.117	1.7	0.0
P. putida	G11.57	1.7	0.0
pseudomonad	GR3.5	1.7	0.2
P. fluorescens	G25.54	1.3	0.0
P. putida	29.420	1.0	0.0
P. putida	G2.10	0.7	0.0
P. fluorescens	G11.46	0.3	0.0
P. putida	GR12.7	0.0	1.0
pseudomonad	86.65	0.0	0.0
P. putida	GR2.11	0.0	0.0
pseudomonad	GR8.5	0.0	0.0
pseudomonad	GR8.8	0.0	0.0
pseudomonad	GR25.97	0.0	0.0
Pseudomonad	L41.3	0.0	0.0
LSD		4.8	0.5

zones, expressed as a clear zone around bacterial colonies, were measured after 5 days of growth. The inhibition test was performed in triplicate using a completely randomized block design.

Fluorescent pigment and cyanide detection

Opine-utilizing bacteria were streaked on PAF medium and incubated for three days at room temperature. The production of a yellow-green pigment which fluoresces under UV light was evaluated.

The cyanide production was evaluated using the technique described by Bakker and Schippers (1987). Positive scores were given when paper soaked in picric acid solution became brown to reddish.

Traits associated with pathogenesis

Hypersensitive reaction on tobacco was evaluated by injecting 1 mL of a Log 8–9 bacterial suspension into well-developed leaves of tobacco. Controls were inoculated with sterile distilled water. Potato soft-rot reaction was tested by inoculating potato disks with a loopful of bacteria. Controls were touched with a sterile loop. The presence of pectolytic enzymes was evaluated with PVC medium (Schaad, 1988).

Results

Growth of *E. carotovora* subsp. *carotovora* was more affected than the growth of *A. tumefaciens* by the opine-utilizing bacteria evaluated (Table

Table 2. Inhibition zones (mm) induced by rhizobacteria towards various fungal pathogens of potato on PAF medium

Strain number	*Botrytis* sp	*Colletotrichum coccodes*	*Verticillium*		*Phoma exugua*	*Fusarium*		
			dahliae	*albo-atum*		*sambucinum*	*solani*	*oxysporum*
4.6	7.7	3.0	9.5	3.0	3.0	4.0	3.0	1.2
212	5.7	4.0	3.7	3.0	2.3	0.2	3.3	0.3
203	5.0	9.7	9.5	7.3	3.5	0.3	2.0	2.3
211	5.0	1.3	1.7	0.7	1.7	0.7	3.7	1.0
1.9	5.0	3.3	2.3	2.0	3.3	1.3	2.0	0.8
G11.46	4.7	2.0	0.5	0.7	1.0	1.7	1.3	0.7
61.9A	4.3	1.7	2.3	1.3	2.3	1.0	2.3	2.3
86.65	3.3	0.3	0.0	0.0	0.0	0.5	1.0	0.0
G25.34	3.3	0.0	0.7	0.3	0.3	0.0	2.0	0.0
GR25.97	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29.420	3.0	0.0	0.0	0.0	0.0	0.2	0.3	0.0
L41.3	3.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
G3.9	3.0	0.0	0.3	0.7	1.0	0.0	0.7	0.0
GR8.17	2.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
G2.10	1.7	0.0	0.0	0.0	0.0	0.0	0.3	0.0
G25.54	1.7	0.0	0.0	0.0	0.0	0.0	0.3	0.0
GR7.4R2	1.3	0.0	0.0	0.0	0.0	0.0	0.3	0.0
G11.57	1.3	1.0	0.0	0.0	1.0	0.3	1.3	1.0
GR17.4	1.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
GR25.5	1.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
GR8.8	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GR12.2	0.7	0.0	0.0	0.0	0.0	0.0	1.3	0.0
GR12.7	0.7	1.0	1.7	1.0	0.0	0.2	0.7	1.0
GR20.5	0.7	0.0	0.0	0.0	0.0	0.0	1.3	0.0
GR19.1	0.3	0.0	0.0	0.0	0.0	0.0	0.3	0.0
GR3.5	0.0	1.0	0.7	0.0	0.0	0.8	2.0	0.0
L16.33	0.0	1.0	1.0	0.5	1.0	0.0	0.0	0.2
GR25.6	0.0	0.0	0.7	0.0	0.0	0.0	1.0	0.0
G12.117	0.0	0.2	0.0	0.2	0.0	0.0	0.7	0.0
GR2.11	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0
GR20.3	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
GR8.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LSD	1.1	0.8	0.4	1.1	0.3	0.6	0.5	0.5

1). Seventy-eight percent of the bacteria tested inhibited *E. carotovora* subsp. *carotovora*, while 19% were active against *A. tumefaciens*. Fifty-two percent of the strains which inhibited *E. carotovora* subsp. *carotovora* had an inhibition zone of more than 3 mm compared to only 3% for the *A. tumefaciens* antagonists. The maximum inhibition zone was 11 mm.

With fungal pathogens, 6% of the opine-utilizing strains inhibited *V. dahliae* with an inhibition zone of more than 7 mm (Table 2). However, most strains did not inhibit this pathogen. Seventy-eight percent of the bacteria inhibited the growth of *Botrytis* sp., with 40% producing an inhibition zone of more than 3 mm. For the other fungi, between 31 to 44% of the strains demonstrated antibiosis, but most had an inhibition zone of less than 3 mm. The maximum inhibition zone was 10 mm.

Two strains (4.6 and 203) inhibited all fungi and bacteria tested. Strains 212, 211, 1.9, G11.46 and 61.9A inhibited all fungi and *E. carotovora* subsp. *carotovora*, but not *A. tumefaciens*. Most strains were only active against specific pathogens.

Production of a fluorescent pigment on PAF medium was observed for 84% of the bacteria tested (Table 3). However, there was no relation between antagonistic potential and fluorescent pigment production.

None of the bacteria induced hypersensitive reaction on tobacco. Three induced potato soft rot, and two of these were pectolytic pseudomonads. Four strains produced cyanide on media containing glycine (Table 2).

Discussion

The opine-utilizing rhizobacteria studied here share physiological traits reported to be associated with known PGPR including antibiosis *in vitro*, production of cyanide, and fluorescent pigment production.

In this study, PAF medium was used to detect iron regulated principles, *i.e.* the fluorescent pigment or antibiotic production, and simultaneously to evaluate bacterial inhibition of potato pathogens. This medium was selected since iron starvation regulates the production of some antibiotics (Gutterson, 1990; Thomashow and Weller, 1990) and is an important factor involved in biological control (Gutterson, 1990; Schippers, 1988). In addition, it has been demonstrated with fewer strains that the addition of iron to PAF medium nullified the inhibition zone in the case of *E. carotovora* (C J Beauchamp, Ph D thesis, Université Laval, Ste-Foy, Québec, Canada, 1989).

Using a similar medium, Thomashow and Weller (1990) reported that phenazine was the primary factor involved in the inhibition of *Gaeumannomyces graminis* var *tritici*, and that *in vitro* activity was linked to *in vivo* inhibition of the pathogen. In this study, the production of fluorescent pigment did not obligatorily relate to inhibition of pathogens, since some strains which did not produce fluorescent pigment also suppressed pathogen growth. Even though PAF medium did not allow the discernment of the active principle, it may be useful in the first screening steps to identify potential PGPR or

Table 3. Properties of opine-utilizing rhizobacteria

Strain	Production of			
	Fluorescent pigment	Potato soft rot	Polygalacturonase	HCN
203	+	+	+	+
211	+	+	+	−
4.6	+	+	−	+
1.9	+	−	−	+
G11.46	+	−	+	+
212, 29.420, 61.9A, 86.65 G2.10, G3.9, G11.12, G11.57 G12.117, G25.34, G25.54 GR2.11, GR7.4R, GR8.5 GR8.17, GR12.2, GR12.7				
GR20.5, GR25.5, GR25.6 GR3.5, GR8.8, GR25.97	+	−	−	−
L16.33, L41.3	−	−	−	−

biological control agents. As reported for indirect-acting PGPR (Kloepper and Schroth, 1978), several of the opine-catabolizing rhizobacteria exhibited broad-spectrum antibiosis. *E. carotovora* subsp. *carotovora* and *Botrytis* sp. were antagonized by a greater number of bacteria than the other plant pathogens. Inhibition was observed for strain 203 against *A. tumefaciens*, *C. coccodes*, *P. exigua*, *V. dahliae*, *V. albo-atrum*, and *F. oxysporum*; for strain 4.6 against *V. dahliae* and *F. sambucinum*, for strain 212 against *F. solani* and *P. exugua*, and for strain 61.9A against *F. oxysporum*. In this study, some dinitrogen-fixing pseudomonads, designated by the letters GR or L, were evaluated (Lifshitz *et al.*, 1986; C J Beauchamp, Ph D thesis, Université Laval, Ste-Foy, Québec, Canada, 1989). These strains were only weak inhibitors of the plant pathogens. A similar situation was previously reported for members of the Azotobacteraceae family, which are dinitrogen-fixing bacteria producing fluorescent pigments (Thompson and Skerman, 1979).

The production of cyanide was initially considered as a trait of deleterious bacteria, since large amounts of cyanide may depress root respiration and indirectly impair nutrient uptake (Schippers *et al.*, 1987). However, cyanide production may also induce plant defence mechanisms, which may improve plant protection against pathogens and therefore have a beneficial effect on plant growth (Voisard *et al.*, 1989). It appears that the amount of cyanide produced is important to determine the ability of a strain to be deleterious or beneficial for plant growth (Alström and Burns, 1989). An analysis of the amount of cyanide produced was not conducted for the bacteria found here to be positive for this character.

Only a few of the opine utilizers tested inhibited the growth of *A. tumefaciens*. This is in accordance with previous results (Digat, 1983). In particular, it has been reported that the siderophore pseudobactin has only a temporary inhibition effect on *Agrobacterium* sp. Pronounced inhibition of *A. tumefaciens* was found only with strain 203. Unfortunately, because this pseudomonad also exhibited traits characteristic of plant pathogens, it may not be considered as a potential biological control agent.

In contrast with opine-catabolizing agrobacteria, only a few of the strains in this study have pathogenic traits. The induction of the hypersensitive reaction, a trait of many plant pathogenic bacteria, was not expressed by the potential antagonists studied here. Among the ten strains exhibiting the strongest antagonism, namely 203, 211, 4.6, 1.9, G11.46, 212, 61.9A, G25.34, GR12.7 and L16.33 (Table 1 and 2), three strains induced potato soft rot. Two of these strains (203 and 211) were isolated from crown-gall tumors (Beaulieu *et al.*, 1983), while one (4.6) was obtained from a potato tuber. The ability to macerate potato tuber tissue and pectinase production are indicative of pathogenic potential found in bacterial soft rotters.

The expression of antagonism on a given medium depends on three basic parameters: the mode of action of the antagonistic principle, the intensity with which this principle is produced, and the susceptibility of the target pathogen to this principle. These three parameters interact to generate potential biocontrol agents with a narrow or broad spectrum of activity. Situations may arise under field conditions, whereby protection may be obtained against a major pathogen or a complex of DRM. Potential PGPR or biological control agents should be chosen accordingly.

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