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Association of pectolytic fluorescent pseudomonads with postharvest rots of onion

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Summary. Five isolates of pectolytic fluorescent pseudomonads were obtained from a rotted onion bulb and identified as *Pseudomonas marginalis*. At both 4 and 25°C, all isolates caused soft rot to detached plant parts of onion and to carrot, celery, cucumber, pepper, spinach, tomato and turnip (but not garlic). They did not however cause any symptoms in living plants of these same species. These results suggest that the onion isolates are a postharvest pathogen which is not destructive in the field but becomes a threat to fresh vegetables stored at low-temperature. Analysis of cellulosolytic and pectic enzymes revealed that pectic lyases, but not polygalacturonases, pectin methyl esterases and cellulases were produced in culture by each isolate.

Key words: postharvest pathogens, *Pseudomonas marginalis*, soft rot.

Introduction

Pseudomonas fluorescens is a nutritionally and physiologically heterogeneous species (Stanier *et al*., 1996) consisting of a diversity of ecological groups, isolated from different sources such as plant tissue (Sands and Hankin, 1975; Surico and Iacobellis, 1978; Liao and Wells, 1987b; Aysan *et al*., 2000), soil (Sands and Hankin, 1975; Wang and Kelman, 1982; Daniel and Fergal, 1992) and sewage (Sands and Hankin, 1975). Some *P. fluorescens* strains cause postharvest soft rots of plant crops during storage and marketing (Brocklehurst and Lund, 1981; Misaghi and Donndelinger, 1983; Liao and Wells, 1987b; Hassan, 1996). These strains are thought to macerate plant tissues by producing pectic enzymes that degrade pectin in the middle lamella. The most common pectic enzyme secreted by *P. fluorescens* is pectate lyase (PL) (Liao *et al*., 1988) which cleaves the α-1,4-glycosidic linkages in pectic polymers by β-elimination and generates products with a 4,5-unsaturated galacturonosyl residue at the non-reducing end (Collmer *et al*., 1988).

Fourteen bacterial isolates were isolated from an onion bulb which did not show any external disease symptoms but contained rotted tissue in the middle. On the basis of cultural, morphological, biochemical and physiological characteristics, 5 of the bacteria isolated were provisionally assigned to pectolytic fluorescent pseudomonads, 3 red pigmented *Serratia marcescens*, one to non-pigmented *Serratia* sp. and 5 to *Klebsiella oxytoca*.

This study was undertaken to characterize the five pectolytic fluorescent pseudomonads and to evaluate their ability to cause soft rot in detached plant parts as well as in living plants of various

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vegetables. The production of pectinases and cellulases by these isolates was also investigated.

Materials and methods

Onion specimen and bacterial isolation

An onion bulb without external disease symptoms but with two yellow cores in the centre (Fig. 1) was obtained from a vegetable market in the El-Haram area, Giza Governorate, Egypt. The bulb was taken directly to the laboratory where the rotted tissue was separated, washed carefully with sterile distilled water and ground in a sterile mortar under aseptic conditions. A loopful of the suspension obtained was streaked onto Petri dishes containing nutrient agar (NA) or the selective medium CVP (Cuppels and Kelman, 1974). After incubation at 28°C for 48 h, all the colonies on NA and all colonies exhibiting pectolytic activity on CVP were picked up, purified on NA and stored on NA at 4°C until further characterization.

Bacterial identification

Five of the pectolytic isolates (On1, On1A, On1H, On1I and On2A) which under UV light (254 nm) showed a green-yellow fluorescence on King's medium B (KB) (King *et al.,* 1954) were identified following the methods described by Lelliott *et al*. (1966), Palleroni (1984) and Lelliott and Stead (1987) for the determination of fluorescent pseudomonads. Identification was also performed with the Biolog identification system (Biolog Inc., New York, NY, USA). The ability of the isolates to oxidize different carbon sources was tested and assimilation profiles were analysed by MLITM System software (Biolog) for bacterial identification. In addition, isolate growth at 4 and 40°C in Luria-Bertani (LB) agar and broth (static culture) was recorded after 7 days of incubation. Growth on agar medium was determined visually, that in broth by measuring the increase of optical density at 600 nm.

Fig. 1. An onion bulb containing two rotted cores from which the pectolytic fluorescent bacteria were isolated.

Cells grown in LB and flagella were observed by transmission electron microscopy (TEM). After incubation for 48 h, the cells were mounted on a copper grid coated with Formvar and carbon, and stained with 1% aqueous uranyl acetate for 1–2 min. The flagella were then examined in a Zeiss EM10 (Germany) apparatus operating at 60 kV.

Pathogenicity tests

Seeds of carrot (*Daucus carrota*), celery (*Apium graveolens)*, cucumber (*Cucumis sativus)*, garlic (*Allium sativum),* onion (*Allium coepa)*, pepper (*Capsicum annuum)*, spinach *(Spinacia oleracea*), tomato (*Lycopersicon esculentum)*, tobacco (*Nicotiana tabacum xanthi)* and turnip (*Brassica rapa)* were purchased from the Ministry of Agriculture, Egypt.

Seeds were sown in trays (30×60×6 cm) containing clay soil. Two weeks after sowing, six seedlings of each species were transferred to a 20-cm diameter plastic pot containing clay soil. All pots were placed on a bench maintained at room temperature, and watered as required to keep the soil moist but not wet.

Three weeks after potting, each isolate was inoculated into six seedlings of each plant species (three seedlings through the leaves and three through the stem). Inocula were prepared from 24-h-old KB cultures incubated at 28°C. Cells were suspended in sterile distilled water and adjusted to the required concentration by measuring OD_{600} and calculating the cell number from the calibration curve. Cells were inoculated using a 1-ml sterile disposable hypodermic syringe with a 0.25 G needle. Each seedling received approx. 3×10^7 cfu through the leaves, or 0.7×10^6 cfu through the

stem. Control seedlings were inoculated with sterile distilled water.

Tissue maceration

Healthy cucumber, pepper and tomato fruits were surface-sterilized in a 1% solution of sodium hypochlorite, washed with sterile distilled water and inoculated with bacterial suspensions of the onion isolates. Each isolate was inoculated into 6 fruits from each plant species, which were then placed in 2 sterile plastic boxes (3 plants per box). One box was incubated at 4°C and the other at 25°C. Control fruits were inoculated with sterile distilled water and incubated at the same two temperatures.

Discs about 1.0 cm thick and 0.8 cm in diameter were cut from the roots of carrot and turnip, the bulbs of garlic and onion, cucumber fruits and potato tubers. Four discs from each plant species were placed in a sterile Petri dish, three discs were inoculated with the bacterial growth, and one disc was used as the control. Petioles of celery, spinach and turnip were placed in sterile dishes and inoculated with the bacterial suspension. Each isolate was inoculated into two identical sets of dishes, each containing petioles or discs of different plant species. One set of dishes was incubated at 4°C, the other at 25°C. Discs and fruits were inoculated following the method of El-Hendawy *et al*. (2002).

Screening for pectic enzymes

Petri dishes containing LB agar plus 0.5% sodium polypectate (P-1879; Sigma, St. Louis, MO, USA) were used for detection of pectic enzymes. Dishes were incubated at 28°C for 3 days. Excretion of pectic enzymes into the medium was indicated by a colourless halo around the colony after flooding with a saturated solution of copper acetate (Keen *et al*., 1984).

Pectin methyl esterase (PME) was assayed by measuring the decrease of pH in a reaction mixture containing citrus pectin (P-9135; Sigma), and the culture supernatant as a source of the enzyme as described by Duncan (1969). Pectic lyase and hydrolase activity was determined by the thiobarbituric acid method (Albersheim *et al*., 1960).

Screening for cellulases

Production of cellulases was determined using a minimal salt medium (Chatterjee *et al*., 1979) containing K_2HPO_4 (0.7%), KH_2PO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.02%), $(NH_4)_2SO_4$ (0.1%), pH 7.0 and supplemented with yeast extract (0.1%), carboxymethyl cellulose (3%) and agar (1.5%) (Liao and Wells, 1987a). Dishes were streaked with bacteria and incubated at 28°C for 3 days, then flooded with Congo red (0.1%), left for 15 min and bleached with 1 M NaCl. Cellulase activity was revealed by the appearance of a halo surrounding the bacterium.

Results

Bacterial isolation and identification

Cells of all 5 isolates were Gram negative, rod shaped, motile by means of polar flagella (Fig. 2). All the isolates were oxidase positive and levan negative; they produced arginine dihydrolase and macerated potato discs. None of the isolates induced a hypersensitive response on tobacco leaves (Table 1). Additional tests revealed that isolates produced 2-ketogluconate, nitrate reductase and acid from sucrose, but not lecithinase from egg yolk. All the isolates grew at 4°C, but none grew at 40°C. They liquefied gelatin and did not hydrolyse starch. They also utilized acetate, succinate, propionate, malate, malonate, lactate, citrate, ribose, manni-

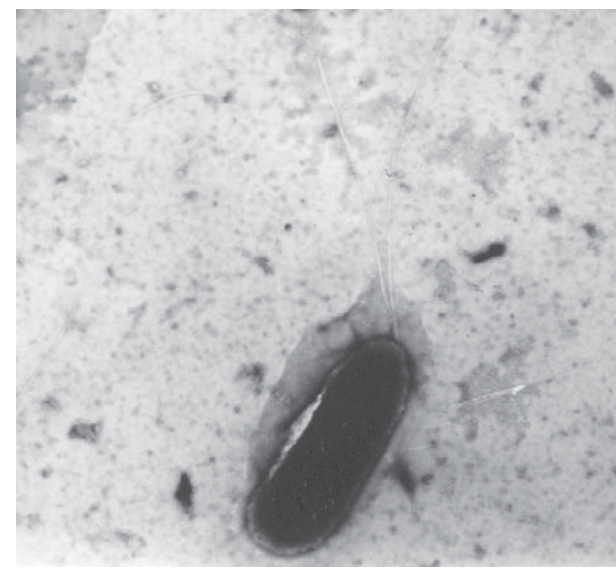

Fig. 2. Polar flagella of isolate On1 grown in Luria-Bertani (LB) broth under static conditions for 48 h (×10,000).

Table 1. Grouping[a] of green-fluorescent pseudomonads by the LOPAT scheme and reactions obtained from the onion isolates.

Group	Presumptive (LOPAT) character					Confirmatory character				Species
	Levan type colonies	Oxidase reaction	Potato rot	Arginine dihydro-lyase	Tobacco hyper-sensitivity	2-keto-gluconate production	Egg yolk reaction	Nitrate reduction	Acid from sucrose	
Ia	+	-	-	-	+	-	-	-	+	*P. syringae*
Ib	-	-	-	-	+	-	-	-	+	*P.s.* subsp. *savastanoi; P. delphini*
II	-	-	+	-	+	-	-	-	-	*P. viridiflava*
III	-	+	-	-	+	-	-	d	-	*P. cichorii; P. agarici*
IVa	+	+	+	+	-	+	+	+	+	*P. marginalis* (pectolytic *P. fluorescens)*
IVb	-	+	+	+	-	+	d	d	d	*P. fluorescens*
Va	-	+	-	+	-	+	+	-	-	*P. tolaasii,* and some saprophytic pseudomonads
Vb	+	+	-	+	-	+	d	d	d	*P. fluorescens* and some other saprophytic pseudomonads
Onion isolates	-	+	+	+	-	+	-	+	+	

[a] Data presented by Lelliott and Stead (1987) from Lelliott *et al*. (1966).
d, Result varies with isolate.

tol, D-xylose, L-arabinose, L-rhamnose, glucose, D-mannose, D-galactose, fructose, sucrose, trehalose, gluconate, saccharate and aconiate, but they did not utilize D-arabinose, D-fructose, maltose, cellobiose, lactose, inuline, D-tartrate or L-tartrate.

Other characteristics of onion isolates, and those of *Pseudomonas fluorescens* biovars reported by Palleroni (1984) are presented in Table 2.

Macerating ability

To determine whether the onion isolates not only grew at 4°C but also macerated plant tissue at that temperature, each isolate was inoculated into two identical sets of detached parts of the vegetables tested. One of the sets of plant parts was incubated at 25°C (optimal bacterial growth temperature for maceration), the other at 4°C. The onion isolates macerated discs of carrot roots, cucumber fruits, onion bulbs and turnip roots and petioles of celery, spinach and turnip at both 4 and 25°C (Table 3). At 25°C all plant parts were macerated within 4 days after inoculation, while at 4°C complete maceration was achieved within 10 days. Tomato fruits were completely macerated when incubated at 25°C for 4 days, but at 4°C, only water soaked areas appeared around the site of inoculation after 7 days.

Garlic discs or cucumber and pepper fruits were not macerated by any of the isolates at either incubation temperature.

Pathogenicity tests

None of the onion isolates induced any rot symptom on living plants of the species tested after leaf or stem inoculation, nor was *P. fluorescens* isolated from any living plants.

Production of pectinases and cellulases

Of all the enzymes tested, only pectic lyase was produced by all 5 onion isolates.

Discussion

Five pectolytic fluorescent pseudomonads were isolated from an onion bulb and identified by morphological and physiological characteristics as *P. marginalis*. However, some of the characteristics of these isolates were different from those report-

Table 2. Characteristics of soft-rotting fluorescent pseudomonads isolated from onion and those reported for *Pseudomonas fluorescens* biovars by Palleroni (1984).

Characteristic	*P. fluorescens* biovar					Onion isolates
	I	II	III	IV	V	
Levan production	+	+	-	+	-	-
Lecithinase (egg yolk)	+	±	+	+	d	-
Nitrate reduction	-	+	+	+	-	+
Tween 80 hydrolysis	d	-	d	d	d	-
Blue nondiffusible pigments	-	-	-	+	-	-
Carbon sources used for growth:						
L-Arabinose	+	+	d	+	d	+
Sucrose	+	+	-	+	d	+
Saccharate	+	+	d	+	d	+
Propionate	+	+	d	+	+	+
Butyrate	-	d	d	+	d	+
Sorbitol	+	+	d	+	d	+
Adonitol	+	-	d	-	d	-
Propylene glycol	-	+	d	-	d	+
Ethanol	-	+	d	-	d	+

+, Positive reaction.
-, Negative reaction.
d, 11–84% strains are positive.

Table 3. Macerating ability of five isolates of fluorescent pseudomonads (On1, On1A, On1H, On1I, On2A) isolated from onion bulb.

Plant part	Maceration index [a, b]									
	On1		On1A		On1H		On1I		On2A	
	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C
Discs of:										
Carrot roots	1.5	4	1	3.5	1.0	3	1	3	1	3.5
Cucumber fruits	5	5	5	5	5.0	5	5	5	5	5
Garlic bulb	0	0	0	0	0.0	0	0	0	0	0
Onion bulb	2.5	5	2	4.5	2.0	4.5	2	4	2	5
Potato tubers	5	5	5	5	5.0	5	5	5	5	5
Turnip roots	4.5	5	4.5	5	4.0	5	4	5	4	5
Petioles of :										
Celery	2.5	4.5	2.5	4.5	2.0	4.5	2	4	2	4.5
Spinach	5	5	5	5	5.0	5	5	5	5	5
Fruits of :										
Cucumber	0	0	0	0	0.0	0	0	0	0	0
Pepper	0	0	0	0	0.0	0	0	0	0	0
Tomato	1	3	0.5	2.5	0.5	2	0.5	2	0.5	2

[a] Maceration index based on a scale from 0 (no maceration) to 5 (maceration of the whole inoculated disc, petiole or fruit).
[b] Results at 25°C were recorded 2 days after inoculation, those at 4°C 7 days after inoculation.

ed in the literature for *P. marginalis*. The onion isolates in this study did not produce levan and lecithinase. This failure of some *P. marginalis* strains to form levan from sucrose has already been reported (Misaghi and Donndelinger, 1983; Liao and Wells, 1987b). Moreover, unlike the findings from our study, lecithinase production from *P. marginalis* was reported to be variable by Palleroni (1984). Lelliott *et al*. (1966) divided green fluorescent pseudomonads by the LOPAT scheme into five groups. Of these groups Group IV, containing soft rot strains that were either positive or negative for levan, positive for oxidase and arginine dihydrolase and negative for the hypersensitivity test on tobacco leaves, was further subdivided into two subgroups: (IVa) containing the levan-positive strains, for which they retained the name *P. marginalis*, and (IVb) containing the levan-negative strains, and which they defined as pathogenic strains of the *fluorescens*/*putida* complex of *Pseudomonas*. The characteristics of the onion isolates were more closely related to *P. fluorescens* biotype II (Palleroni, 1984) than to *P. putida*.

No cellulase activity was detected by any of the isolates. The failure of pectolytic fluorescent pseudomonads to show cellulase activity was reported by Sands and Hankin (1975) and by Liao and Wells (1987b). In this study, however, it was not determined whether cellulase was not produced, or was cell-bound.

The onion isolates showed pectolytic activity on CVP and on LB medium containing 0.5% sodium polypectate. Further analysis detected neither polygalacturonases (PG) nor pectin methyl esterases (PME) in the culture supernatants of any of the isolates, though it did detect pectic lyase activity.

Only a few *P. fluorescens* strains produce PGs (Nasuno and Starr, 1966; Zucker and Hankin, 1970; Hildebrand, 1971), pectin lyase (Schlemmer *et al*., 1987; Sone *et al.,* 1988 ; Nikaidou *et al*., 1992) and PME (Nasuno and Starr, 1966), whereas pectate lyase has long been reported to be produced by fluorescent soft rot bacteria (Fuchs, 1965; Nasuno and Starr, 1966; Zucker and Hankin, 1970; Hildebrand, 1971; Liao, 1989; Elumalai and Mahadevan, 1995; Liao *et al*., 1997).

Although the pathogenicity of *P. marginalis* to garlic has been reported at least once (Kuropatwa *et al*., 1997), none of the onion isolates in our study macerated garlic discs. That *P. marginalis* did not cause soft rot on garlic was shown experimentally by Wright and Grant (1998). Since garlic has long been known for its antibacterial activity against various Gram-positive and Gram-negative bacteria (Singh and Shukla, 1984; Rod *et al*., 1989*;* Elsom *et al*., 2000; Kyung and Lee, 2001; Kyung *et al*., 2002 ; Lee *et al*., 2003), the failure of the onion isolates to macerate garlic discs could be due to this bactericidal characteristic of garlic.

All isolates grew at 4°C and caused soft rot in detached plant parts of carrot, celery, cucumber, onion, pepper, spinach and tomato at 4 and 25°C. In contrast, none of the isolates were pathogenic to living plants of the same species. Admittedly the soft rot tests on plant parts were carried out under controlled conditions, in which oxygen and humidity levels may well be highly suitable for the growth and secretion of tissue-macerating enzymes secreted by the pathogen (De Boer and Kelman, 1978; Maher and Kelman, 1983; Ko *et al*., 2002), while the inoculation of living seedlings was carried out in the open air, where the environment may have suppressed growth and enzyme production by the pathogen.

In Liao and Wells (1987a), pectolytic xanthomonads associated with postharvest rots of plant crops macerated detached plant parts of those crops but were not pathogenic to the same plants when grown in the greenhouse. The ability of our isolates from onion to grow and macerate plant parts at 4°C combined with their failure to macerate living plant tissue is evidence suggesting that the onion isolates are a postharvest pathogen that does not damage plants grown in the field but is a threat to fresh vegetables stored at low temperature. This would mean that storage methods other than cold storage are required to protect plants against this pathogen.

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