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Phenotypic heterogeneity of *Pseudomonas corrugata* strains from southern Italy

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V. CATARA, L. GARDAN AND M.M. LOPEZ. 1997. Fifty-five strains of *Pseudomonas corrugata* isolated in southern Italy were characterized phenotypically and compared with 23 strains of different origins. At least two main cultural types with rough or smooth colonies were observed. Strains with rough colonies produced a diffusible pigment in culture. On the basis of their nutritional profiles, *Ps. corrugata* strains formed a distinct phenon most closely related to fluorescent *Pseudomonas* spp. isolated from tomato pith necrosis-diseased plants. Three major groups of strains were differentiated within the *Ps. corrugata* phenon on the basis of utilization of 2-ketogluconate, meso-tartrate, hystamine, DL-glycerate and induction of a hypersensitive reaction on tobacco. Some *Ps. corrugata* strains belonging to group 1 and 3 which did not produce pigment in culture produced IAA in a colorimetric test. Variability in the serological reaction of the Italian strain was observed. None of the three antisera utilized reacted with all strains. Some strains isolated from diseased plants from the same greenhouse showed different nutritional profiles and reacted with different antisera. Fifteen lipopolysaccharide (LPS) patterns were observed. Strains were divided into two groups on the basis of their protein profiles. The heterogeneity which had already been observed in a world-wide study on *Ps. corrugata* was confirmed in strains from this restricted area.

INTRODUCTION

Pseudomonas corrugata Roberts and Scarlett is the causal agent of tomato pith necrosis (TPN) (Scarlett *et al.* 1978). The disease occurs on tomato world-wide (Bradbury 1987) and the bacterium has also been isolated from pepper (Lopez *et al.* 1988) and chrysanthemum with pith necrosis (Fiori 1992), soil (Scortichini 1989), water (Scarlett *et al.* 1978), symptomless alfalfa roots (Lukezic 1979), and wheat rhizosphere (Roberts and Brewster 1991). It can cause significant losses, particularly in tomatoes grown in unheated greenhouses where a great difference in temperature between night and day, and high humidity, seem to favour the development of the disease (Scarlett *et al.* 1978). These conditions occur in Sicily, one of the major Italian tomato producing areas, where TPN losses of up to 90% have been observed (Catara and Albanese 1993). Variability in phenotypic characters has been reported among *Ps. corrugata* strains isolated in different countries, although these studies were carried out on a limited number of strains or characters. In a study carried out on biochemical and serological features of a world-wide collection of 128 *Ps. corrugata* strains, variability was observed in biochemical characteristics as well as in pigment production and colony morphology (Siverio *et al.* 1993). High heterogeneity was also observed in reactions to three antisera and the presence of at least 11 lipopolysaccharide profiles but no correlation to the origin of isolation was reported (Siverio *et al.* 1993). Although these data showed a great variation within this species, it is not known if such variation could also occur in a restricted geographical region among strains indigenous to the island of Sicily. During the course of an investigation, several greenhouses were surveyed in Sicily and *Ps. corrugata* was isolated from different samples. The aim of this investigation is to study the phenotypic characteristics of *Ps. corrugata* strains isolated in this area and to

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compare them with strains of different origin (Italian or broader).

MATERIALS AND METHODS

Bacterial strains

Different sets of strains were used in this study, depending on the test performed. The first set included 55 strains of *Ps. corrugata* isolated in Sicily (southern Italy) from diseased tomato plants or from soil of infected greenhouses, and eight strains came from other areas of Italy (Table 1). The second group included 15 *Ps. corrugata* strains from different countries (Table 1). The third group contained seven type strains of species of phytopathogenic fluorescent *Pseudomonas* and five strains of pathogenic fluorescent *Pseudomonas* spp. isolated from TPN-affected tomato plants in different countries (Table 1). The strains were maintained on nutrient agar (Difco, Detroit, USA) at 4 °C during the course of the experiments. Bacteria were preserved in 15% glycerol at −70 °C for long-term storage.

Pathogenicity tests

All *Ps. corrugata* strains and TPN-associated fluorescent *Pseudomonas* spp. strains were tested for hypersensitivity reaction (HR) on tobacco (Klement and Goodman 1967) and pathogenicity on tomato. The inoculum was prepared from a 24 h culture by suspending bacterial cells in sterile distilled water up to a concentration of 10^8 cfu ml^{-1}. Tomato plantlets cv. Arletta grown in trays were inoculated when they were 20 cm high and had at least three true fully expanded leaves. Three plants per strain were inoculated by injecting 50 μl of a bacterial suspension at the axil of the first true leaf. Control plants were inoculated with sterile distilled water, or with a *Ps. corrugata* reference strain (NCPPB 2445). The plants were placed in a growth chamber with 16/8 h day/night photoperiod and temperatures of 25 and 18 °C, respectively.

Table 1. Bacteria analysed in this study

Species	Strain*	Origin	Source‡
Pseudomonas corrugata†	(A1); (F1, P1, 3B, 3C); (D1, D2) (1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.10, 1.11); (2.1, 2.2, 2.3); (3.1); (4.1, 4.2, 4.3.t, 4.4.t, 4.5.t); (5.1.t, 5.2.t); (6.1.t); (7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8); (8.1, 8.2, 8.3); (9.1); (10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, 10.10, 10.11, 10.12, 10.13, 10.14)	Italy (Sicily)	IPVCT
	59a, 60b, 60d, 98a, 98b;	(Sardinia)	M. Fiori
	B002, B003, B021;	(Latium)	M. Scortichini
	82.23.6, 83.83.4, 86.16.9;	France	L. Gardan
	J375	Germany	S. Köhn (IVIA)
	1.1.6, 592.4.4, 1113.5, 536.7.1, T6, 5.4, 614.5.3, 632.2, 632.5;	Spain	IVIA
	8895;	New Zealand	ICMP (IVIA)
	2445	UK	NCPPB
Pseudomonas spp.	10435	Canada	CFBP
(TPN-associated)	10529, 10530	France	CFBP
	18, 60	Italy	IPVCT
Ps. aeruginosa	2466	USA	CFBP
Ps. cichorii	2101	UK	CFBP
Ps. fluorescens	2102	USA	CFBP
Ps. fuscovaginae	2065	Japan	CFBP
Ps. marginalis	1387	USA	CFBP
Ps. putida	2066	UK	CFBP
Ps. tolaasii	2068	UK	CFBP

* Strains underlined were studied with biotype 100 strips and included in numerical taxonomy.

† *Pseudomonas corrugata* strains underlined were studied for AIA production and whole-cell protein profiles. All strains isolated in Italy were also studied in serology and LPS profiles. Sicilian strains were grouped by brackets according to the greenhouse of isolation.

‡ Name of the researcher or institution, and in brackets the source from which we received the strains. IPVCT, Istituto di Patologia Vegetale, Catania, Italy; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; Collection Francaise des Bactéries Phytopathogènes, Angers, France.

The plants were maintained in polyethylene bags for the first 48 h. The plants were observed for 15 d for the development of symptoms.

Morphological, biochemical and physiological tests

Colony characteristics, including pigment production, were determined on YPGA (7 g l^{-1} yeast extract, 7 g l^{-1} bactopeptone, 7 g l^{-1} D-glucose and 15 g l^{-1} agar) and NDA (nutrient agar, 1% D-glucose). All strains were characterized by the following tests according to the methods reported by Schaad (1988): fluorescent pigment production on King's medium B (King *et al.* 1954), oxidase reaction, levan production, arginine dihydrolase production, gelatine and esculin hydrolysis, lipolysis of Tween-80. Nitrate reduction was determined as described by Lelliot and Stead (1987) and pectate liquefaction as described by Sutton (1957).

The nutritional profiles of 50 *Ps. corrugata* strains isolated in different countries (indicated in Table 1), five TPN-associated fluorescent *Pseudomonas* spp. and seven type strains of fluorescent *Pseudomonas* spp. (Table 2) were analysed with Biotype 100 strips (Biomerieux, La Balne les Grottes, France). These strips, containing a total of 99 carbon sources (carbohydrates, organic acids and amino acids), were inoculated, incubated at 24 °C and examined after 4 d for growth.

Numerical taxonomy

A total of 110 characters was included in the numerical taxonomy analysis of the 62 above cited strains. The distance matrix was calculated by using the Jaccard coefficient (Sneath and Sokal 1973). Cluster analysis was performed by the unweighted pair group method with averages (UPGMA) (Sneath and Sokal 1973). With the numerical identification programme utilized, it was possible to assign each isolate to a phenon (Lapage *et al.* 1973; Bollet *et al.* 1988). The amount of information for each test was measured by calculating the diagnostic ability coefficient (DAC) at the distance level of interest (Descamp and Véron 1991).

Indolacetic acid production

The ability to produce indolacetic acid (IAA) was investigated *in vitro* by using a colorimetric technique (Gordon and Weber 1951). The 32 strains of *Ps. corrugata* utilized in numerical analysis were inoculated at *ca* 10^5 cfu ml^{-1} and grown with shaking for 48 h at 25°C in 5 ml of King B medium containing 2·5 mmol tryptophan. After centrifugation (10 min, 12 000 g, 4 min), 0·5 ml of culture supernatant fluid was added to 1 ml of modified Salkowski's reagent (100 ml concentrated sulphuric acid; 3 ml 1·5 mol l^{-1} Fe Cl_3; 60 ml H_2O) (Gardan *et al.* 1992a). The tubes were incubated at room temperature for 30 min in the dark, the optical densities were read at 530 nm, and the absorbance spectrum between 370 and 670 nm obtained with a spectrophotometer (Kontron Uvikon, Watford, UK; 941 plus). The amount of IAA was determined by using a previously determined calibration curve (Gardan *et al.* 1992b).

Serology and lipopolysaccharide profiles

All *Ps. corrugata* strains isolated in Italy were evaluated in indirect immunofluorescence (indirect IF) and indirect enzyme-linked immunosorbent assay (indirect ELISA), for their reactions to three antisera: AS-2445, AS-536.7.1 and AS-592.4.4 as described by Siverio *et al.* (1993). The antisera were produced against the *Ps. corrugata* type strain NCPPB 2445 and two Spanish strains, 536.7.1 from tomato and 592.4.4 from pepper, included in this study. As lipopolysaccharides (LPS) have been reported to be determinants of serological variability of *Ps. corrugata*, the LPS profiles of the strains tested in this serological study were also analysed (Siverio *et al.* 1993). Indirect IF was performed as described by De Boer (1990) and doubtful reactions with a very low level of fluorescence, as well as no reaction, were considered negative. Indirect ELISA was performed as described by Siverio *et al.* (1993). In order to compare strains located in different plates, serological reaction (Alarcon *et al.* 1990) was calculated as follows: $SR = [(O.D. - y)/(x - y)] \times 100$, where respectively, O.D., x, and y were the optical density for the strain studied (average of four wells), the positive standard (average of six wells in the corresponding microplate), and the negative standard (average of six wells in the respective microplate). Results were considered negative when the SR was below 50. Both indirect IF and indirect ELISA were performed twice. LPS profiles were obtained by digestion of cell lysate protein with 25 $\mu g\ ml^{-1}$ of proteinase k (Siverio *et al.* 1993) and separation by one-dimensional SDS-PAGE (Laemmli 1970) in 12·5% acrylamide gels. Gels were stained by LPS selective silver staining (Tsai and Frasch 1982).

Electrophoresis of whole-cell proteins

All *Ps. corrugata* strains utilized in numerical taxonomy were characterized by one-dimensional SDS-PAGE of whole-cell proteins according to the method of Laemmli (1970) as described by Jackman (1985). Cells from a 1·5 ml sample of an 18 h culture in King's B broth were harvested by centrifugation and washed twice in sterile deionized water. Whole cell proteins were prepared as described by Bouzar *et al.* (1994a). Protein separation was performed in 10% acrylamide gels. Gels were stained with 0·2% Comassie brilliant blue staining method. Each strain was analysed at least twice.

RESULTS

Pathogenicity tests

All *Ps. corrugata* and fluorescent *Pseudomonas* spp. strains were able to reproduce symptoms of TPN in tomato plants. *Pseudomonas corrugata* strains caused a slight crack of the stem 3 d after inoculation and sometimes, slight browning. In a few plants, production of adventitious roots was observed 15 d after inoculation. The stem in longitudinal section revealed brown water-soaked and/or dry pith with cavities. The strains caused lesions ranging from 1 to 3 cm. Fluorescent *Pseudomonas* spp. strains did not induce external symptoms. The stem sections revealed brown water-soaked discoloration of the pith. No differences in the symptomatology induced by the strains tested were observed.

Phenotypic characteristics of *Ps. corrugata*

The colonies of the majority of *Ps. corrugata* strains studied showed a rough surface and a curly margin, but they could appear umbonate, raised, dry or mucous, cream or yellow in colour on YPGA as well as on NDA. On the same media they produced a diffusible yellow pigment. Three strains isolated in Sicily (8.1; 8.2; 8.3), as well as some strains from other origins, produced whitish-cream, smooth-surfaced colonies that did not produce any pigment (Table 2). Results of production of pigment in the two media were comparable except for the two strains 86.16.9 and 82.23.6 which produced pigmentation only on YPGA (Table 2). The intermediate phenotype observed for strains that produced smooth colonies and the diffusible pigment was not observed for Sicilian strains. No *Ps. corrugata* strains fluoresced on KB medium,

Table 2. Phenotypic characteristics of *Pseudomonas corrugata* strains*

Strain	Cluster analysis†	Protein profile‡	Serology§	IAA¶	Pigmentation> YPGA	NDA
83.83.4	dp	1	(2, 3)	+	−	−
1.1.6	1A	1	(3)	+	−	−
B021	1A	1	3	+	−	v
8.1; 8.2; 8.3	1A	1	3	+	−	−
82.23.6	1B	1	nt	+	+	−
592.4.4; 9.1; 3.B; 3C; 1.1; 1.4; 1.11; P1	1B	1	3	−	+	+
2445; 2.1; 2.2	2	2	1	−	+	+
10.2; 10.3; 4.3.t; 4.5.t; B002;	2	2	2	−	+	+
7.2; 10.6;	2	2	3	−	+	+
98a	2	2	1, 2	−	+	v
D1	2	2	1, 2	−	+	+
3.1; 4.1; 4.2; 5.1.t; 5.2.t; 6.1.t; 7.6; 7.8; 10.14;	2	2	2, 3	−	+	+
60b; 10.8; 10.12; A1;	2	2	0	−	+	+
8895; 1113.5	2	2	(0)	−	+	+
536.7.1	3A	2	2	+	+	v
J375	3B	2	(0)	+	−	−
86.16.9	3C	2	nt	+	+	−
5.4	3C	2	(3)	+	−	−
614.5.3	3C	2	(1)	+	−	−
632.2; 632.5; T6	3C	2	(0)	+	−	−

* In this table, *Ps. corrugata* strains analysed with all the techniques were included.
† Cluster analysis groups obtained by numerical analysis of all 110 tests performed. The numbers define the groups obtained at distance level 0·08 and the capital letters the subgroups obtained at distance level 0·05; dp = distinct phenotype.
‡ SDS-PAGE whole cell protein profiles. Two different groups were defined on the basis of two differences in the profile.
§ IF-I and/or ELISA-I reaction with antiserum 1 = AS-2445, 2 = AS-536.7.1, 3 = AS-592.4.4. 0 = did not react with any antisera; nt = not tested. Results in brackets were not obtained in this study but were reported from Siverio *et al* (1993).
¶ IAA production titrated with Salkowski's reagent. Strains were considered positive when a concentration more than 25 μg ml^{-1} was measured, and the maximum peak of absorbance was between 538 and 540 nm.
>Diffusible pigment production on media YPGA and NDA. Results on YPGA were utilized in numerical taxonomy, v = strains that did not give the same result in different plating.

all were oxidase positive, levan negative, produced arginine dihydrolase, hydrolysed aesculin, and reduced nitrates beyond NO_2, and none liquefied pectate. Variable results were obtained with gelatine hydrolysis, lipolysis of Tween-80, and hypersensitive reaction on tobacco leaves. The *Ps. corrugata* strains tested with biotype 100 strips utilized 55 out of 99 substrates.

Numerical taxonomy

The dendrogram (Fig. 1) displays the relative distance of the 62 strains including 50 *Ps. corrugata* strains and other pseudomonads. At distance level 0·14, three phenons and six distinct strains were observed. Phenon I included two type strains (*Ps. fluorescens* and *Ps. tolaasii*). Phenon II included all but one strain of the 50 *Ps. corrugata* strains tested and one strain of *Pseudomonas* spp. TPN; phenon III included four fluorescent strains classified as *Pseudomonas* spp., all isolated from TPN-diseased plants, one in Sicily, two in France and one in Canada. At distance level 0·08, one distinct strain (*Pseudomonas* sp. IPVCT 60) and three groups were recognized within the *Ps. corrugata* phenon. Groups 1 and 2 contained *Ps. corrugata* strains from different geographical origins and hosts, including strains from Sicily, whereas group 3 did not contain any Sicilian strains (Fig. 1). At distance level 0·05, it was possible to delineate two subgroups in group 1 (1A, 1B) and three in group 3 (two distinct strains considered to form subgroups 3A and 3B and subgroup 3C) (Fig. 1).

Characterization of phenons and single strains was based on 44 biochemical tests selected by the highest DAC (Table 3). The strains clustered in phenon III (fluorescent *Pseudomonas* spp.) were more closely related to those of phenon II (*Ps. corrugata*) than to the reference *Pseudomonas* species, and were distinguishable from strains of phenon II because of the production of fluorescent pigment on KB and utilization of D-sorbitol. *Pseudomonas corrugata* strain 83.83.4 was not included in phenon II because it could be differentiated by at least six biochemical tests from the other strains of *Ps. corrugata*.

Seven biochemical tests were selected to differentiate groups and subgroups delineated at distance level 0·08 and 0·05 inside the *Ps. corrugata* phenon (Table 4). Group 1 differed from groups 2 and 3 because of the utilization of 2 keto-D-gluconate, *meso*-tartrate, histamine and DL-glycerate. Groups 2 and 3 were separated by the results to tobacco hypersensitivity. Only two characteristics discriminated groups 1A and 1B (gelatine hydrolysis and presence of diffusible pigment on LPDA). The same characteristics discriminated within group 3A, 3B and 3C.

IAA production

Concentrations of indolic products, titrated by colorimetry, produced by *Ps. corrugata* strains ranged from 19 to 180 $\mu g\ ml^{-1}$. Only the strains producing the maximum peak of absorbance at about 538–540 nm (probable presence of IAA) were considered positive (Table 2). Three strains from Sicily (8.1; 8.2; 8.3) produced values of 180 $\mu g/ml$. Concentrations of more than 100 $\mu g\ ml^{-1}$ were observed for seven isolates with different origins, four Spanish 614.5.3, 1.1.6, 632.2, 632.5 and three French strains 83.83.4, 82.23.5, 86.16.9.

Serology

None of the antisera utilized reacted with all the 66 strains (Table 5) tested, and seven strains did not react with any of the antisera (10.7; 10.8; 10.9; 10.12; Al; 60b; 60d). Calculated SR by indirect ELISA of the strains with the different antisera revealed that none of the strains reacted with more than one antiserum, six reacted with AS2445, eight with AS536.7.1, and 32 with AS592.4.4. In indirect IF, eight strains reacted with AS2445, 29 with AS536.7.1 and 40 with AS592.4.4; four strains reacted with the first two antisera and 14 with AS536.7.1 and AS592.4.4. Some strains with the same origin (i.e. from the same greenhouse) reacted with different and/or no antisera.

SDS-PAGE and selective staining for LPS resolved a profile with a high number of bands for each strain. LPS profiles obtained from the strains analysed were compared on the basis of the number, intensity, and relative mobility of the electrophoretic bands after conversion of the images in scans with a densitometer. The strains tested presented 15 different smooth type profiles (Table 5) of which eight were identical to that observed by Siverio *et al.* (1993). None of the strains showed a rough type profile (i.e. absence of O-chains). Strains that in indirect IF reacted with the same fluorescence of the homologous strain, and in indirect ELISA with an SR of more than 90, shared the same LPS profiles as the homologous strain. None of the strains showed the same profile as strain 592.4.4. High variability in LPS was observed among strains from different greenhouses.

Protein profiles

SDS-PAGE analysis of whole-cell extracts of the *Ps. corrugata* strains resulted in reproducible profiles made up of a large number of discrete bands. Strains from Italy as well as from different origins showed a high degree of similarity of the bands in the profile. The presence of some bands, or groups of bands, in the profiles distinguished at least two groups of isolates (Fig. 2). One group of 22 strains showed a different position of four bands in the zone between 66 and 45 kDa compared to the remaining strains, and the presence of a low relative mobility band almost at the base of the gel. Only 15 of the 22 strains were analysed for their biochemical and physiological characteristics in numerical taxonomy, and

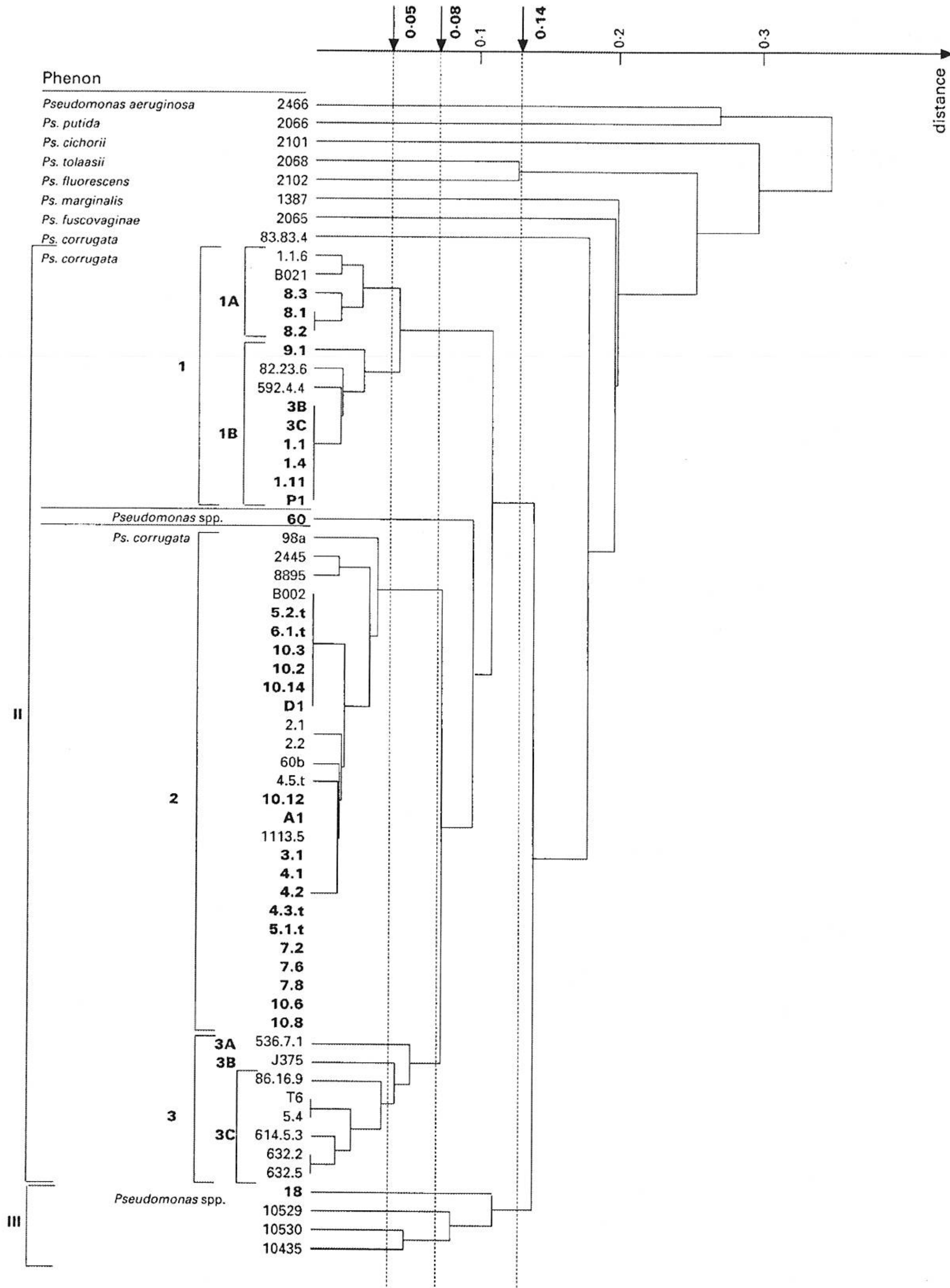

Fig. 1 Dendrogram of similarity based on the distance of 50 *Pseudomonas corrugata* strains, four TPN fluorescent *Pseudomonas* and six fluorescent *Pseudomonas* different species. Sicilian strains are in boldface

Table 3. Characteristics that differentiate three phenons and six isolated phenotypes at the distance $d = 0{\cdot}14$*

Characteristics	Phenons			Distinct phenotypes†					
	I	II	III	1 2066	2 2466	3 2065	4 1387	5 2101	6 83.83.4
Fluorescence	+	–	+	+	+	+	+	+	–
D-Sorbitol	+	–	+	–	–	–	+	–	–
Itaconate	+	–	d	+	+	–	+	–	–
D-Lyxose	+	+	+	–	–	–	+	–	–
Nitrate reduction	–	+	+	+	+	–	–	–	+
D(+) Xylose	d	+	+	–	–	+	+	+	–
Tobacco HR	–	d	–	–	–	+	–	+	–
Sucrose	d	+	+	–	–	–	+	–	–
D(+) Trehalose	+	+	+	–	–	+	+	–	–
myo-Inositol	+	+	+	–	–	–	+	+	+
5-keto-D-gluconate	+	–	–	–	–	–	+	–	–
Benzoate	d	–	d	+	+	–	–	–	–
L(+) Arabinose	d	+	+	–	–	+	+	+	+
N-Acetyl-D-glucosamine	+	+	d	–	+	–	+	–	+
Levan production	–	–	d	–	–	–	+	–	–
Trigonelline	–	+	+	+	–	+	+	+	+
D(+) Galactose	+	+	+	–	–	+	+	+	+
D(+) Mannose	+	+	+	–	–	+	+	+	+
Esculin hydrolysis	–	–	–	–	–	–	+	+	–
D(+) Arabitol	+	+	+	–	+	+	+	+	–
D(–) Arabitol	+	–	–	–	–	–	–	–	–
Xylitol	+	–	–	–	–	–	–	–	–
D-Mannitol	+	+	+	–	+	+	+	+	–
Adonitol	+	–	–	–	–	–	–	–	–
2-Keto-D-gluconate	+	d	d	+	+	–	+	–	+
meso-Tartrate	+	d	d	+	–	+	–	+	+
i-Erythritol	d	–	–	–	–	–	+	–	–
Ethanolamine	d	+	+	+	+	+	+	–	+
Histamine	–	d	–	+	+	–	–	–	+
Phenylacetate	–	–	d	+	–	–	–	–	–
L(+) Tartrate	–	–	–	+	–	–	–	+	–
Gelatin hydrolysis	d	d	d	–	+	–	+	+	–
D(+) Cellobiose	–	–	–	–	+	–	–	–	–
D-Saccharate	+	+	–	+	–	+	+	+	+
Mucate	+	+	+	+	–	+	+	+	+
trans-Aconitol	+	+	+	+	–	+	+	+	+
D-Glucuronate	+	+	+	+	–	+	+	+	+
D-Galacturonate	+	+	+	+	–	+	+	+	+
Gentisate	–	–	–	–	+	–	–	–	–
Putrescine	+	+	+	+	+	–	+	+	+
DL-β-Hydroxybutyrate	+	+	+	+	+	+	+	–	+
L-Tyrosine	+	+	+	+	+	+	+	–	+
Arginine dihydrolase	+	+	+	+	+	+	+	–	+
Pectate liquefaction	–	–	–	–	–	–	+	–	–

* + = 90% or more of the strains are positive; – = 90% or more of the strains are negative; d = 10–89% of the strains are positive.
† Isolated strains are listed in Table 4.

Table 4. Characteristics that differentiate groups among *Pseudomonas corrugata* strains*

Tests	Group or subgroup							
	1	1A	1B	2	3	3A	3B	3C
2-Keto-D-gluconate	+	+	+	−	−	−	−	−
meso-Tartrate	+	+	+	−	−	−	−	−
Histamine	+	+	+	−	−	−	−	−
DL-Glycerate	+	+	+	−	d	+	−	d
Gelatin hydrolysis	d	−	+	−	d	+	+	−
Tobacco HR	d	d	+	+	−	−	−	−
Lypolisis of Tween-80	−	−	−	d	−	−	−	−
Pigment on YPGA	d	−	+	+	d	+	−	d
No. of strains	14	5	9	27	8	1	1	6

* + = 90% or more of the strains are positive; − = 90% or more of the strains are negative; *d* = 10–89% of the strains are positive.

Table 5. Serological reaction of *Pseudomonas corrugata* strains isolated in Italy by IF-I* and ELISA-I† and LPS patterns‡

Strain	Reaction to antiserum					
	AS 2445		AS 536.7.1		AS 592.4.4	
	IF	ELISA	IF	ELISA	IF	ELISA
2445^{I}; 2.1^{I}; 2.2^{I}; 2.3^{I}	+	+	−	−	−	−
536.7.1^{II}; 10.1^{II}; 10.2^{II}; 10.3^{II}; 10.4^{II}; 10.5^{II}; B002^{II}; B003^{II};	−	−	+	+	−	−
4.3.t^{VI}; 4.4.t^{VI}; 4.5.t^{VI};	−	−	+	−	−	−
592.4.4^{III}; 1.3^{VII}; 1.11^{VII}; P1^{VII}; F1^{VII}; 3B^{VII}; 3C^{VII}; 59a^{VII}; 7.1^{VIII}; 7.2^{VIII}; 10.6^{IX}; 10.10^{IX}; 10.11^{IX}; 8.1^{X}; 8.2^{X}; 8.3^{X}; 9.1^{XII}; B021^{XV};	−	−	−	−	+	+
1.1^{VII}; 1.2^{VII}; 1.4^{VII}; 1.5^{VII}; 1.6^{VII}; 1.7^{VII}; 1.8^{VII}; 1.10^{VII};	−	−	−	−	+	−
98a^{I}; 98b^{I};	+	+	+	−	−	−
D1^{XI}; D2^{XI};	+	−	+	−	−	−
3.1^{IV}; 4.1^{IV}; 10.13^{IV}; 10.14^{VI}; 4.2^{VI}; 5.1.t^{VIII}; 7.3^{VIII}; 7.4^{VIII}; 7.5^{VIII}; 7.6^{VIII}; 7.7^{VIII}; 7.8^{VIII}; 5.2.t^{XIII}; 6.1.t^{XIV};	−	−	+	−	+	+
60b^{V}; 60d^{V}; A1^{V}; 10.7^{VI}; 10.8^{VI}; 10.9^{VI}; 10.12^{VI};	−	−	−	−	−	−

* + Positive: same or less fluorescence than the homologous strain; − negative or doubtful reaction.
† Strain reactions to the different antisera by ELISA-I are expressed as positive (+) or negative (−) on the basis of their serological reaction (SR) described in the text.
‡ LPS patterns for each strain are indicated in superscript roman numbers.

were included in group 1 (14 strains) and the distinct strain 83.83.4 (Table 2).

DISCUSSION

The heterogeneity that has already been observed in a worldwide collection of *Ps. corrugata* strains (Siverio *et al.* 1993) was also confirmed in strains from the restricted area of southern Italy. Furthermore, the investigation revealed the same extent of variability regardless of the geographical or substrate source of the strains. The first characterization of these strains revealed differences in morphology and pigmentation of colonies. As reported by Siverio *et al.* (1993), the presence of at least two cultural types with rough or smooth colonies was observed in *Ps. corrugata* strains. This phenotype seemed to be the least widespread in Sicily, being isolated only from one infected greenhouse. In this study, strains with rough colonies produced diffusible pigment, while not all smooth colonies strains were without pigment. Thus, the presence of an intermediate phenotype is not ruled out. Change from one phenotype to another was never observed in culture during this study. Different results in

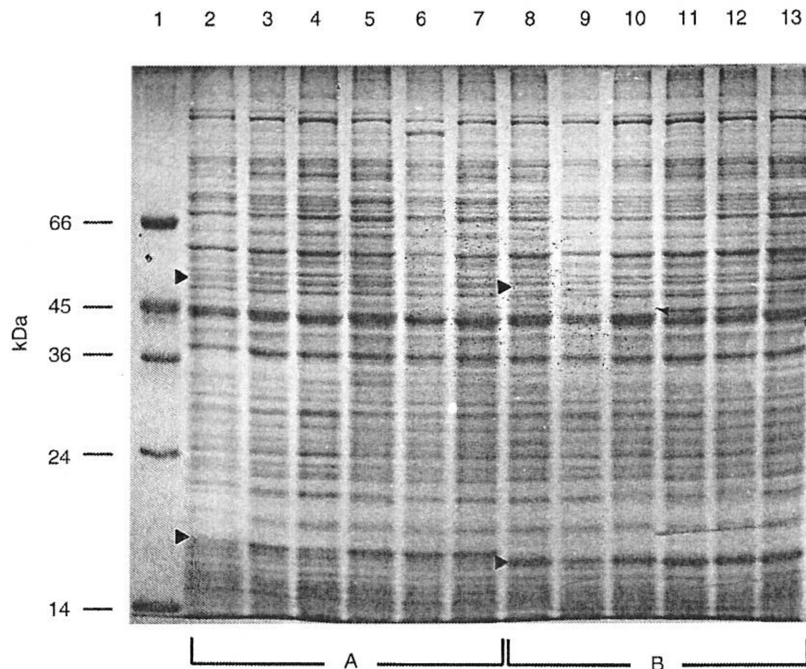

Fig. 2 SDS-PAGE of whole-cell proteins of some *Pseudomonas corrugata* strains. Lane 1 : protein molecular weight markers, from the top to the bottom, serum albumin bovine (66 kDa) ; ovalbumin (45 kDa) ; glyceraldehyde-3-phosphate dehydrogenase (36 kDa) ; trypsinogen (24 kDa) ; α-lactalbumin (14·2 kDa). Lane 2 : 1.1.6 ; lane 3 : B021 ; lane 4 : 8.3 ; lane 5 : 9.1 ; lane 6 : 3B ; lane 7 : 1.1 ; lane 8 : 2.2 ; lane 9 : 60b ; lane 10 : 3.1 ; lane 11 : 7.2 ; lane 12 : J375 ; lane 13 : 614.5.3. Strains are grouped in A (from lane 2 to 7) and B (from lane 8 to 13) on the basis of differences in two areas of the profiles indicated by arrows

pigmentation were observed in the two media (NDA and YPGA) for only a few strains, and may have affected the interpretation of the results based on pigment production.

All the strains tested here were positive to arginine dihydrolase, including those described as negative in other studies. In our investigation, nitrate reduction was always positive and esculin hydrolysis was always negative. Strains were variable, as reported elsewhere, in gelatine hydrolysis and lipase on Tween-80 (Siverio *et al.* 1993 ; Lopez *et al.* 1994). Furthermore, Biotype 100 strips defined the nutritional profile of *Ps. corrugata* in standard conditions with 99 carbon sources, as has been done with nutritional galleries for other plant pathogenic species (Gardan *et al.* 1991 ; Gardan *et al.* 1992a). The results obtained allowed us to define the substrates utilized by *Ps. corrugata* and to exclude those with variable results. This could be useful for the preparation of a selective medium for the recovery of bacterium from soil or other complex habitats. Numerical analysis permitted the grouping of *Ps. corrugata* into three major groups and several subgroups. It was observed that *Ps. corrugata* strains from different sources can have identical biochemical profiles and that strains from the same greenhouse can have different profiles. Group 1, formed by 14 *Ps. corrugata* strains, was clearly distinguishable from the other strains by cluster analysis of biochemical characteristics and SDS-PAGE of whole-cell proteins. This group differed from other groups because it utilized 2-keto-D-gluconate, *meso*-tartrate, and histamine. SDS-PAGE of whole-cell protein showed two regions of the profile which were completely different in this group, thus representing an easy method for distinguishing strains. The strains in this group reacted with the AS-592.4.4 with indirect ELISA and/or indirect IF, and some also with AS-536.7.1, while the other strains showed a more variable reaction. Strain 83.83.4 was considered as a distinct phenotype in the dendrogram, while it shared all the above-mentioned characteristics.

We found that 15 strains of *Ps. corrugata* produced IAA ; this has been well demonstrated for several pseudomonads (Fett *et al.* 1987 ; Surico and Iacobellis 1990). Spectrophotometric analysis revealed that these strains showed a maximum absorbance peak at 538–540 nm, thus, within the range of indolic products. Other strains gave a peak at 500–520 nm, but the chemical substance equivalent to this wavelength was not investigated. The IAA-producing strains were those of group 1A of numerical taxonomy, one strain in group 1B, and strains of group 3 ; none of the strains in group 2 produced IAA. None of the strains which were positive in this test produced diffusible pigment on YPGA (character utilized in numerical taxonomy), except one strain in group 3 and one in group 1B which produced pigment on YPGA, but not on NDA.

In cluster analysis of nutritional characteristics, all the *Ps. corrugata* strains from different geographical origins, including Italian strains, formed a group that was clearly separated from the other pseudomonads studied. The most closely related group to *Ps. corrugata* comprised fluorescent pseudomonads isolated from tomato plants affected by pith necrosis in different countries. Despite their origin (Canada, France

and Italy), these four strains represented a homogeneous group whose nutritional profile differed from the other pseudomonads tested. Nevertheless, one of the TPN fluorescent strains (IPVCT 60) was located inside the *Ps. corrugata* phenon and this could be justified by the relationship between the two groups. *Ps. corrugata* has already been described as closely related to some fluorescent pseudomonads on the basis of its phenotypic characteristics (Lukezic 1979), DNA–rRNA homology (De Vos *et al.* 1985) and fatty acid profiles (Stead 1992). Furthermore, all fluorescent TPN-isolated *Ps. corrugata* spp. strains reproduced TPN on tomato plants.

In serological tests, only a few strains showed a reaction with antisera of the same intensity as the homologous strain. More strains were positive in indirect IF than in indirect ELISA. The antiserum with which most strains reacted was AS-592.4.4, but marked serological variability was observed. In fact, strains isolated in Sicily reacted with one, two or none of the antisera utilized. This variability was equally apparent in strains from a restricted origin as in those from different origins. Furthermore, in two instances, strains from the same greenhouse gave different serological reactions. This finding was explained by the high number of LPS profiles observed in the Sicilian strains, because it was previously demonstrated that LPS are the major antigens of *Ps. corrugata* (Siverio *et al.* 1993). The results of LPS profile were only correlated with serology.

Comparison of whole cell protein profiles also helped to provide a better characterization of the bacterium and to indicate that more than one group is present inside *Ps. corrugata*, as occurs for other bacterial species (Vauterin *et al.* 1991; Bouzar *et al.* 1994a). The *Ps. corrugata* strains studied showed high homology in the protein profile, but some elements led to differentiation of at least two different groups. The first group coincided with group 1 of numerical taxonomy which is also characterized by serological homogeneity reacting with AS 592.4.4, although correlation between biochemical and serological characteristics has not often been described.

In conclusion, this bacterium can be identified by few biochemical tests, pathogenicity tests and protein profiling. *Pseudomonas corrugata* can be soil-borne (Scortichini 1989) and seed- (Zutra and Kritzman 1983) or water-transmitted (Scarlett *et al.* 1978). The origin of the variability of the strains of *Ps. corrugata* in Sicily is not clear because of the historical cultivation of tomatoes in this area and because seeds have been imported for many years. The high variability of populations, even in the same greenhouse, calls for other tests, such as those based on molecular biology, to characterize and differentiate the strains in order to localize the inoculum source. *Pseudomonas corrugata* strains isolated in Sicily were analysed with different techniques which have already been used to study the variability of strains at specific level (Bouzar *et al.* 1994b). The results obtained underline the variability in *Ps. corrugata* of the physiological characters, nutritional profile, IAA production, serological reactions and whole-cell protein profiles in the strains isolated in Sicily and those of different geographic origin. Furthermore, strains isolated from diseased plants in the same greenhouse can have a different biochemical protein profile or react with different antisera. Despite the variability observed among strains, the phenotypic characteristics of *Ps. corrugata* clearly distinguish this group from other species.

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