APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1993, p. 1805–1812 Vol. 59, No. 6
0099-2240/93/061805-08$02.00/0

Lipopolysaccharides as Determinants of Serological Variability in *Pseudomonas corrugata*

F. SIVERIO,[1] M. CAMBRA,[2] M. T. GORRIS,[2] J. CORZO,[3] AND M. M. LOPEZ[2]*

Centro de Investigación y Tecnología Agraria, 38080 La Laguna,[1] and Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, 38205 La Laguna,[3] Tenerife, and Instituto Valenciano de Investigaciones Agrarias, 46113 Moncada, Valencia,[2] Spain

Received 3 December 1992/Accepted 31 March 1993

The variation in biochemical and serological features of 128 isolates of *Pseudomonas corrugata* has been studied with 56 isolates from Spain and 72 isolates from other countries. Isolates were analyzed with common diagnostic tests and with the API50CHE system. Variability among isolates for some standard tests usually listed as positive or negative for this species, such as arginine dihydrolase and gelatin hydrolysis, lipase and lecithinase activities, pigment production, and wrinkled colony morphology, was observed. Three antisera were raised against the type strain and two Spanish isolates from tomato and pepper plants. Serological reactions were studied by indirect immunofluorescence and indirect enzyme-linked immunosorbent assay. Eighty-three isolates reacted with a single antiserum, 6 reacted with two antisera, and none reacted with three antisera. Thirty-nine isolates did not react with any of the three antisera. These results suggest that serology will not be a useful method for routine diagnosis of *P. corrugata* unless common antigens can be identified. Electrophoresis and immunoelectrotransfer were used to study the antigens involved. Each antiserum reacted with whole-cell lysates, giving two common bands for *P. corrugata* isolates and other *Pseudomonas* species and a ladder-like pattern characteristic of lipopolysaccharides (LPS). Common bands were not observed after proteinase K treatment. More than 10 LPS patterns were distinguished in 98 isolates after silver staining of polyacrylamide gels. There was no correlation between the geographical origin or host of the isolates and the LPS patterns. A correlation between LPS groups and serological reaction was observed.

Pseudomonas corrugata Roberts and Scarlett has been cited as the causal agent of tomato and pepper pith necrosis (21, 22, 27). The bacterium was described in 1978 in the United Kingdom, after isolations from diseased tomato plants, on the basis of colony morphology and biochemical and pathological characteristics (27). The disease has been reported in many countries in which *P. corrugata* has been considered an opportunistic bacterium or has been cited as the cause of important losses (22, 24). *P. corrugata* has been isolated from water (27), soil (25, 26, 29), symptomless alfalfa roots (23), and rice (33). The lack of rapid and specific methods for the identification of *P. corrugata* makes diagnosis and epidemiological studies difficult.

Morphological, biochemical, and physiological characteristics of isolates of *P. corrugata* from various countries have been reported by several authors. Some of them point out differences in colony aspect (23, 27) or biochemical and physiological variability in some tests (25). The available information about serological characterization of *P. corrugata* is limited to the serological variability among *P. corrugata* isolates observed on tube agglutination (6) and detection of some isolates by enzyme-linked immunosorbent assay (ELISA) (11, 24). Interest in this study of *P. corrugata* lies in the possibility of developing specific methods for detection and identification and contributing to knowledge of the phenotypical heterogeneity of this species.

This paper summarizes the biochemical, physiological, and serological characteristics of 128 isolates of *P. corrugata* from 10 countries and three hosts. The serological variability observed may result from lipopolysaccharide (LPS) variation among isolates. The correlation between LPS groups and serological reactivity was also studied.

MATERIALS AND METHODS

Bacterial cultures. The origins of the 128 isolates of *P. corrugata* studied are given in Table 1. Isolates were checked for purity, were routinely grown on King's B medium (16), and were stored at −70°C. In addition, four saprophytic bacteria isolated from tomato plants and five plant pathogenic pseudomonads (*P. syringae* pv. tomato, *P. solanacearum*, *P. syringae* pv. lachrymans, *P. syringae* pv. savastanoi, and *P. gladioli*) from the Instituto Valenciano de Investigaciones Agrarias collection were used for immunoelectrotransfer (IET) assays.

Pathogenicity test. A suspension of 10^7 CFU/ml in sterile distilled water from 24-h cultures was used as the inoculum. Two tomato plants per isolate were inoculated by injection of approximately 0.2 ml of the bacterial suspension at 10 and 30 cm from the crown. Ten tomato plants injected with sterile water were used as controls. The plants were placed in clear polyethylene bags for 48 h and then were unbagged. The plants were grown in a greenhouse at 20 to 25°C and 60 to 70% relative humidity. After 2 months, external and internal symptoms were observed.

Characterization. All isolates were characterized by the following tests: Gram stain (28), production of fluorescent pigment in King's B medium (16), production of yellow pigment and colony morphology in PYGA (5 g of Bacto Peptone per liter, 5 g of yeast extract per liter, 10 g of glucose per liter, and 20 g of agar per liter), anaerobic growth on Hugh and Leifson's medium (15) supplemented with 10 g of glucose per liter, production of levan (20), nitrate reduction (7), arginine dihydrolase (30), oxidase reaction (17), lipase

* Corresponding author.

TABLE 1. Origin of the isolates of *P. corrugata*

Location	Source[a]	Isolates[b]
France	L. Gardan	**80.26.5, 83.83.4**
Germany	S. Köhn	**J.374**, J.375, J.376, **J.609**, J.610, J.611
	K. Naumann and E. Griesbach	V.40, **V.45, Da.do.2**, Da.do.3
Italy	M. Scortichini	Pcl.86, **903FS**, 903PD, **903T**
Japan	H. Kuwata and K. Oikawa	**C1, C2**, C3, C4, E1, E2, **F1**, F2, **G1**, G2
New Zealand	ICMP	7634, 8889, **8890, 8891**, 8894, 8895, **8896, 8898, 9303**
Spain	IVIA	536.1.1, 536.5.1, **536.6.2, 536.7.1, 536.10.2, 542.1.1, 542.1.3, 576.7.1**, 588.2.1, **588.3.1**, 614.1, **614.4.1, 614.5.3**, 632.2, **632.5, 712.2.a, P. Cor. 1**, P. Cor. 2 **T.6, T.7**, T.12, 1.1.3, **1.1.6, 1.1.11**, 1.2.2, **1.2.3, 2.1**, 2.7, 2.8, 5.3, **5.4**, 5.8, **9.2, 9.3**, 9.4, 9.5, **12.3, 12.4**, 12.5, 12.6, **12.7, 14.1**, 14.2, 14.3, 14.4, **14.6**, 1113.2, 1113.5, **29.1.r**, 29.2.r, 29.3.r, **29.1.l**, 29.2.l, 29.3.l. **592.4.4, 592.5.4** (*Capsicum annuum*)
Sweden	K. Olsson and P. Persson	**53, 54**, 55, 56
Switzerland	J. Vogelsanger and R. Grimm	6, 76, **113**, 121, 489, 490, **492**, 496, 501, 504, **580**
United Kingdom	NCPPB	**2445**, 2447, **2449**, 2450, **2451**, 2455, **2456, 2457**, 2458, **2903**
United States	W. P. Bond and L. L. Black	Pc.1, **Pc.2, Pc.3, Pc.11**, Pc.12
	J. B. Jones	JPc.2, **JPc.3**, JPc.4
	F. L. Lukezic	759, 792. **299, 313** (*Medicago sativa*)

[a] ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.

[b] Isolates with boldface names were used for the API50CHE system. Unless otherwise noted (in parentheses), the host was *Lycopersicon esculentum*.

with Tween 80 (20), gelatin hydrolysis (20), lecithinase activity (20), and accumulation of poly-β-hydroxybutyrate (20).

The carbohydrate metabolism of 64 selected isolates (in boldface in Table 1) was studied with the API50CHE system. The isolates were grown on nutrient agar at 25°C for 24 h, and a suspension of 10^8 CFU/ml in sterile distilled water was used for the tests. Inoculation was performed as described by the manufacturer, and the strips were incubated aerobically at 25°C for 48 h.

Preparation of antigens. Bacterial antigens were prepared with strain 2445 NCPPB (type strain) and with the Spanish isolates 536.7.1 from tomato plants and 592.4.4 from pepper plants. Bacterial cultures were grown for 48 h on King's B medium, and suspensions of 10^9 CFU/ml were grown in phosphate-buffered saline (PBS [NaCl, 8 g/liter; $Na_2HPO_4 \cdot 12H_2O$, 2.7 g/liter; $NaH_2PO_4 \cdot 2H_2O$, 0.4 g/liter], pH 7.2) were used. Somatic antigens were obtained by treating the suspensions at 100°C for 2 h.

Antiserum production. Three antisera (AS-2445, AS-536.7.1, and AS-592.4.4) were prepared. Female rabbits (Californian-New Zealander cross) ca. 2 kg in weight were intramuscularly injected weekly for 4 weeks with 2 ml of a 1:1 emulsion of bacterial antigen with Freund's incomplete adjuvant. Subsequently, the rabbits were immunized with three intravenous injections of 1 ml at 3-day intervals. The animals were bled 3 days after the final injection. The antisera were sterilized by filtration and were stored at −70°C.

IF-I. The indirect immunofluorescence (IF-I) technique described by De Boer (8) was used in multiwell microscope slides. Slide mounting fluid (0.9 ml of glycerol per ml, 0.1 ml of PBS per ml, 1 mg of *p*-phenylenediamine per ml) was used to prevent fading. The preparations were examined with a Leitz Orthoplan microscope. At least two replicates per isolate and antiserum were made. A reaction was considered positive only when the bacterial cell was stained and was considered doubtful when a very low level of fluorescence was observed (Table 2).

ELISA-I *and calculation* of serological reaction. Indirect ELISA (ELISA-I) was used to determine antiserum titer and serological relationships. Four wells of a polystyrene Immunoplate Maxisorp plate (Nunc) were filled with 200 μl (per well) of a 10^9-CFU/ml bacterial suspension of each isolate in carbonate buffer (1.59 g of Na_2CO_3 per liter, 2.93 g of $NaHCO_3$ per liter [pH 9.6]) and were incubated for 16 h at 4°C. The plates were washed three times with PBS-Tween 20 buffer (0.5 ml of Tween 20 per liter in PBS). The wells were filled with 190 μl of rabbit antiserum per well at a working dilution in PBS and were incubated for 2 h at 37°C. Microplates were washed as before and 190 μl of peroxidase-conjugated goat anti-rabbit immunoglobulin (Boehringer Mannheim) was added at a 1/10,000 dilution in PBS. After incubation for 2 h at 37°C, the plates were washed, 200 μl of substrate (1 mg of *o*-phenylenediamine per ml, 0.4 μl of 30% [wt/vol] H_2O_2 per ml in phosphate-citrate buffer [5.35 g of citric acid per liter, 8.85 g of $Na_2HPO_4 \cdot 2H_2O$ per liter in distilled water; pH 5.0]) was added per well, and the solution was incubated for 5 min in darkness at room temperature. The reaction was stopped by the addition of 50 μl of 3N H_2SO_4 to each well. The A_{450} was measured in a Titertek Multiskan MKII reader (Flow). A standard positive control was introduced in every plate to make comparisons between different plates feasible. Six wells per plate were coated with a 1:1:1 mixture of a 10^9-CFU/ml carbonate buffer suspension of the three homologous isolates. These wells were subsequently treated with a 1:1:1 mixture of the three antisera at working dilutions. As a standard negative control, the same mixture of bacterial suspension was assayed in six wells per plate against a *Xylophilus ampelinus* rabbit antiserum. In order to make comparisons among plates, the serological reaction (SR) (2) of the different *P. corrugata* isolates was calculated as $SR = [(x - y)/(z - y)] \cdot 100$, where x was the optical density for the studied isolate (average of four wells), z was the optical density for the standard positive (average of six wells in the corresponding microplate), and y was the optical density for the standard negative (average of six wells in the respective microplate). Results were considered negative when the SR was below 50.

TABLE 2. Reaction of 128 *P. corrugata* isolates by IF-I[a]

Isolate	Reaction to antiserum			Isolate	Reaction to antiserum			Isolate	Reaction to antiserum		
	2445	536.7.1	592.4.4		2445	536.7.1	592.4.4		2445	536.7.1	592.4.4
2445	+++	−	−	542.1.3	−	−	+++	29.2.r	−	−	d
536.7.1	−	+++	−	576.7.1	−	+++	−	29.3.r	−	−	−
592.4.4	−	−	+++	588.2.1	−	−	d	29.1.l	−	−	−
80.26.5	d	d	−	588.3.1	+++	−	−	29.2.l	−	−	−
83.83.4	d	++	++	614.1	−	−	−	29.3.l	−	−	−
J.374	+++	−	−	614.4.1	−	−	d	592.5.4	−	−	+++
J.375	−	d	−	614.5.3	++	++	d	53	+++	++	d
J.376	−	−	−	632.2	d	−	d	54	+++	−	−
J.609	+++	−	−	632.5	−	d	−	55	+++	++	−
J.610	+++	−	−	712.2.a	+++	−	−	56	+++	−	−
J.611	+++	−	−	P. Cor. 1	+++	−	−	6	d	−	−
V.40	+++	d	−	P. Cor. 2	+++	d	−	76	−	−	−
V.45	+++	d	−	T.6	−	−	d	113	+++	−	−
Da.do.2	+++	−	−	T.7	+++	−	−	121	++	−	−
Da.do.3	+++	−	−	T.12	−	−	−	489	+++	d	−
Pcl.86	d	+++	−	1.1.3	d	d	+++	490	+++	d	d
903FS	d	+++	−	1.1.6	−	−	d	492	+++	−	−
903PD	−	−	+++	1.1.11	−	−	++	496	−	−	−
903T	−	d	−	1.2.2	−	−	d	501	−	−	−
C1	+++	++	−	1.2.3	−	−	−	504	+++	d	d
C2	+++	d	−	2.1	−	+++	−	580	++	d	−
C3	+++	d	−	2.7	−	+++	−	2447	d	d	−
C4	+++	−	−	2.8	−	+++	−	2449	−	+++	−
E1	+++	−	−	5.3	−	−	−	2450	+++	d	−
E2	+++	−	−	5.4	−	−	−	2451	−	d	−
F1	−	−	−	5.8	d	−	−	2455	−	+++	−
F2	−	−	−	9.2	+++	d	d	2456	−	−	−
G1	−	−	++	9.3	+++	d	−	2457	+++	d	−
G2	−	−	++	9.4	+++	d	−	2458	++	++	−
7634	d	−	d	9.5	+++	−	−	2903	−	+++	−
8889	+++	−	−	12.3	−	−	−	Pc.1	−	−	d
8890	+++	−	−	12.4	−	−	−	Pc.2	−	−	+++
8891	d	+++	−	12.5	−	−	−	Pc.3	−	−	−
8894	−	−	−	12.6	−	−	−	Pc.11	−	−	−
8895	−	−	−	12.7	−	−	d	Pc.12	−	−	−
8896	−	−	−	14.1	−	+++	−	JPc.2	−	−	−
8898	++	−	−	14.2	−	+++	−	JPc.3	−	−	−
9303	+++	−	−	14.3	−	+++	−	JPc.4	−	−	−
536.1.1	−	−	d	14.4	−	+++	−	759	++	d	d
536.5.1	−	+++	d	14.6	−	−	++	792	d	−	−
536.6.2	−	−	−	1113.2	d	d	−	299	−	−	−
536.10.2	−	+++	−	1113.5	d	d	−	313	+++	−	−
542.1.1	d	−	+++	29.1.r	−	−	d				

[a] Symbols: +++, same fluorescence as homologous strain; ++, less fluorescence than homologous strain; d, doubtful reaction; −, nonfluorescent bacterial cell.

Preparation of whole-cell bacterial protein samples, electrophoresis, and silver staining. The 65 selected isolates (underlined in Table 3) were used for IET. Preparation of the extracts was performed as previously described (1). The isolates were grown for 24 h at 25°C, and 0.7 ml of a 10^8-CFU/ml bacterial suspension in distilled water was mixed with 0.3 ml of sample treatment buffer (17.5 ml of Tris-HCl [0.5 M; pH 8], 2 g of sodium dodecyl sulfate, 5 ml of 2-mercaptoethanol, 10 ml of glycerol, 0.35 mg of bromophenol blue), and this was boiled for 5 min in a heating block. Aliquots of each extract were subjected to proteinase K hydrolysis (14). All of the samples were centrifuged to remove debris and were stored at −20°C until needed. Electrophoresis was performed by the method of Laemmli (19) in 14% acrylamide 0.7-mm-thick slab gels. Samples of 15 μl per well were applied for IET, and samples of 30 μl per well were applied for silver staining. Electrophoresis was performed until the tracking dye reached the end of the gel. Silver staining was performed at least twice (32) with the 69 selected isolates underlined in Table 4.

IET. After electrophoresis, the samples were electrotransferred to polyvinylidene difluoride membranes (Immobilon; Millipore) with a Millipore Milliblot-SDE system. The transfers were made according to the manufacturer's instructions. The polyvinylidene difluoride membranes were then blocked with Tris-HCl-buffered saline (TBS [20 mM Tris-HCl, pH 7.5, plus 29.2 g of NaCl per liter]) with 5 ml of Tween 20 per liter for 30 min (4). To confirm the results, some samples were transferred to nitrocellulose membranes (Millipore) by the method of Towbin et al. (31). The membranes were soaked in diluted antisera (AS-2445, 1/50,000; AS-536.1.1, 1/16,000; AS-592.4.4, 1/10,000 in TBS with 0.05% Tween 20) and were incubated at room temperature with gentle shaking for 2 h. Subsequently, the membranes were washed three times with 0.5 ml of Tween 20 per liter in TBS for 5 min and were incubated in 1/5,000 alkaline phosphatase-conjugated

TABLE 3. Serological reaction of 128 strains of *P. corrugata* by ELISA-I and IF-I[a]

Reaction	Strains reacting with antiserum: 2445	536.7.1	592.4.4
Positive by ELISA-I and IF-I	2445, J.374, J.609, J.610, J.611, V.40, V.45, Da.do.2, Da.do.3, **C1**, C2, C3, C4, E1, E2, 8889, 8890, 8898, 9303, 588.3.1, P. Cor. 1, P. Cor. 2, T.7, 9.2, 9.3, 9.4, 9.5, 54, **55**, 56, 113, 489, 490, 492, 504, 2450, 2457, 313 (38)	536.7.1, Pcl.86, 903FS, 8891, 536.5.1, 536.10.2, 576.7.1, 2.1, 14.1, 2449, 2455, 2903 (12)	592.4.4, **83.83.4**, 903PD, G1, G2, 542.1.1, 542.1.3, 1.1.3, 1.1.11, 14.6, 592.5.4, Pc.2 (12)
Positive by ELISA-I and negative by IF-I	(0)	(0)	8896, 1.1.6, 1.2.2, 5.4, 29.1.r, 29.2.r, 29.3.r, 29.1.l, 29.2.l, 29.3.l, Pc.1, Pc.3, JPc.2, JPc.3, JPc.4 (15)
Negative by ELISA-I and positive by IF-I	**614.5.3**, 712.2.a, **53**, 121, 580, **2458**, 759 (7)	**83.83.4**, **C1**, **614.5.3**, 2.7, 2.8, 14.2, 14.3, 14.4, **53**, **55**, **2458** (11)	(0)
Negative by ELISA-I and IF-I	80.26.5, J.375, J.376, 903T, F1, F2, 7634, 8894, 8895, 536.1.1, 536.6.2, 588.2.1, 614.1, 614.4.1, 632.2, 632.5, T.6, T.12, 1.2.3, 5.3, 5.8, 12.3, 12.4, 12.5, 12.6, 12.7, 1113.2, 1113.5, 6, 76, 496, 501, 2447, 2451, 2456, Pc.11, Pc.12, 792, 299 (39)		

[a] Underlined strains were used for IET. Strains in boldface reacted with two antisera. Numbers in parentheses are number of strains reacting.

goat anti-rabbit immunoglobulin (Boehringer Mannheim) in TBS with 0.05% Tween 20. After being washed as before, the membranes were soaked in 0.1 M Tris, pH 9.6, and were developed (5) with 0.1 M Tris, pH 9.6, instead of Veronal buffer. The reaction was stopped by washing with distilled water.

TABLE 4. Isolates grouped by LPS patterns studied by IET or silver staining

LPS pattern	Isolate(s)[a]
I	2445[I], J.374[I], J.609[I], V.45[I], Da.do.2[I], C1[I, II], 8890[I], 8898[I], 9303[I], 588.3.1[I], P. Cor. 1[I], T.7[I], 9.2[I], 9.3[I], 54[I], 113[I], 492[I], 2457[I], 313[I]
II	536.7.1[II], 903FS[II], 536.10.2[II], 576.7.1[II], 2.1[II], 2.7[II], 2.8[II], 14.1[II], 14.2[II], 14.3[II], 14.4[II], 2449[II], 2455[II], 2903[II]
III	592.4.4[III], 542.1.1[III], 542.1.3[III], 14.6[III], 592.5.4[III], Pc.2[III]
IV	J.375, J.376, 7634, T.6, T.12, 5.3, 5.4[III], 5.8, 299
V	80.26.5, 1113.2, 1113.5, 6, 76, 121[I], 2456, 632.5
VI	903T, F1, F2, 614.1, 614.4.1, 1.1.6[III], 1.1.11[III], 1.2.2[III], 1.2.3, 12.3, 12.4, 12.5, 12.6, 12.7, 2447, Pc.3[III]
VII	29.1.r[III], 29.2.r[III], 29.3.r[III], 29.1.l[III], 29.2.l[III], 29.3.l[III], Pc.1[III], JPc.2[III], JPc.3[III], JPc.4[III], 759[I]
VIII	Pc.11, Pc.12
IX	501, 2451
X	8895
XI	536.1.1, 536.6.2
XII	792
XIII	8894
XIV	588.2.1
XV	632.2
XVI[b]	83.83.4[II,III], 614.5.3[I,II], 580[I], 2458[I,II]

[a] [I], isolate reacted with antiserum 2445 by IF-I or ELISA-I; [II], isolate reacted with antiserum 536.7.1 by IF-I or ELISA-I; [III], isolate reacted with antiserum 592.4.4 by IF-I or ELISA-I. Isolates without superscripts did not react with any of the three antisera. Underlined isolates were used for electrophoresis and silver staining.

[b] Rough-type LPS pattern.

RESULTS

Pathogenicity test. All of the isolates produced typical *P. corrugata* symptoms: necrosis and pith hollowing. External symptoms were almost nonexistent. At least two of the four inoculated points per isolate showed symptoms of pith necrosis. The lessions were very variable in length, averaging 60 mm.

Characterization. The isolates studied on PYGA showed either wrinkled (36.7%), smooth (43.7%), or intermediate (19.6%) morphology. Seventy-five per cent of the isolates produced a yellow pigment. No isolate produced levan or fluorescent pigment on King's B medium (16) or grew under anaerobic conditions. All of them were positive for nitrate reduction (beyond nitrite), poly-β-hydroxybutyrate accumulation, and oxidase reaction. Variable characters of *P. corrugata* were lipase, lecithinase, arginine dihydrolase, and gelatin hydrolysis. These tests gave the following percentages of positive reactions at 3, 7, and 15 days, respectively: lipase, 58, 74, and 80%; lecithinase, 57, 70, and 78%; arginine dihydrolase, 20, 26, and 67%; and gelatin hydrolysis, 74, 75, and 78%.

Results obtained with the API50CHE system showed that after 48 h of incubation, more than 90% of isolates produced acid from glycerol, L-arabinose, galactose, glucose, fructose, mannose, inositol, mannitol, sucrose, and trehalose; acid was produced by 75 to 90% of isolates from ribose and D-xylose; and acid was produced from D-fucose, D-arabitol, and melibiose by 64, 47, and 8% of the isolates, respectively. The substrates erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, rhamnose, dulcitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, inulin, melezitose, raffinose, starch, glyco-

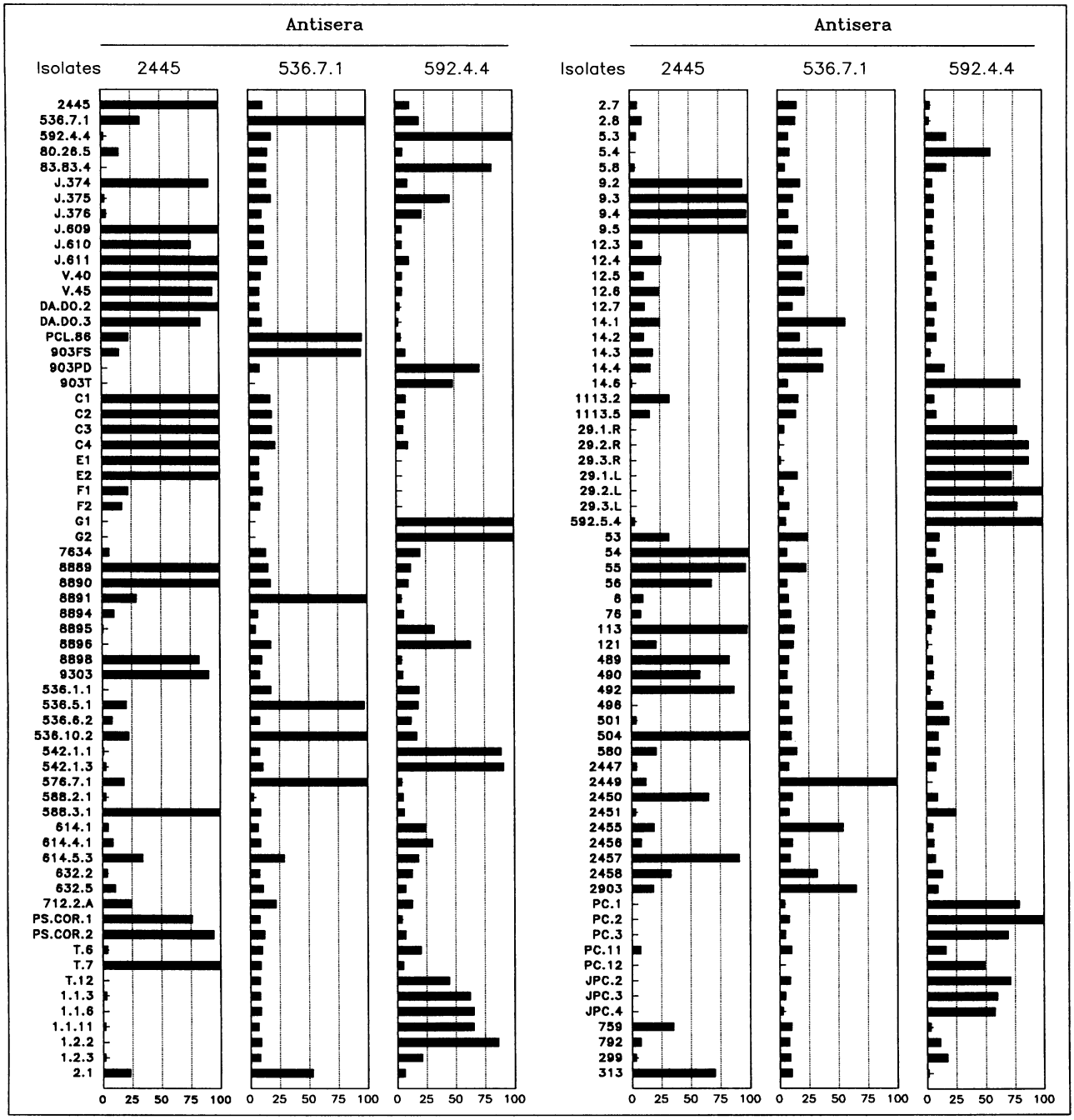

FIG. 1. SRs of isolates. SR = $[(x - y)/(z - y)] \cdot 100$, where x is optical density for the studied strain (mean of four wells), z is optical density for the standard positive of the microplate (mean of six wells coated with a 1:1:1 mixture of the three homologous strains and treated with a 1:1:1 mixture of the three antisera), and y is optical density for the standard negative of the microplate (mean of six wells coated with a 1:1:1 mixture of the three homologous strains, treated with *X. ampelinus* rabbit antiserum).

gen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, L-arabitol, gluconate, 2-ketogluconate, and 5-ketogluconate were not used by any isolate.

Serology. The working dilutions of the three antisera by ELISA-I and IF-I were 1:25,000 and 1:400, respectively, for AS-2445, 1:8,000 and 1:200 for AS-536.7.1, and 1:5,000 and 1:200 for AS-592.4.4.

Table 2 shows the reactions of the 128 isolates in IF-I with the three antisera. A positive reaction was recorded when bacterial cells were fluorescent. Some isolates that presented only fluorescent flagella were considered negative.

Figure 1 shows the calculated SRs by ELISA-I of the isolates with the three antisera. The SR was variable but allowed classification of the isolates into separate groups. Table 3 summarizes the serological responses obtained with both techniques. Each antiserum displayed no cross-reactiv-

ity with either of the two other isolates used to prepare antisera. No antiserum was able to react against all of the isolates, and 39 isolates did not react with any of the antisera produced. Eighty-three isolates reacted with a single antiserum, only 6 isolates reacted with two antisera (in boldface in Table 3), and none reacted with three antisera. AS-2445, AS-536.7.1, and AS-592.4.4 recognized 35, 21, and 18% of the isolates, respectively, by ELISA-I or IF-I.

Electrophoresis, IET, and proteinase K digestion. A characteristic ladder-like pattern of smooth-type LPS was observed by IET in the 19 isolates (underlined in Table 3) that reacted positively in ELISA-I and IF-I with AS-2445. A different pattern was observed in the nine studied isolates that reacted positively in ELISA-I and IF-I with AS-536.7.1. However, with this antiserum, some isolates, such as 2455, did not show the lower-molecular-weight bands of the LPS pattern (Fig. 2B, lanes 5w and p). Another pattern of smooth-type LPS, but with poorly resolved bands, was observed in only four isolates out of seven studied that reacted positively in ELISA-I and IF-I with AS-592.4.4. The other three isolates gave a variable number of bands with no appearance of LPS. Figure 2 shows examples of the patterns observed in the isolates that reacted positively in ELISA-I and IF-I with AS-2445 (Fig. 2A), AS-536.7.1 (Fig. 2B), and AS-592.4.4 (Fig. 2C).

The 65 isolates studied by IET showed a common band about 19 kDa in size with the three antisera, although it was less intense with AS-2445 at the working dilution. Another band with electrophoretic mobility slower than the front was always observed on the transfers made with the Milliblot-SDE system but was not detected by the technique of Towbin et al. (31). These two bands were also detected in *P. syringae* pv. tomato, *P. syringae* pv. lachrymans, *P. syringae* pv. savastanoi, and in three of the four saprophytic *Pseudomonas* species studied, and the bands were not detected in extracts of *P. gladioli* or *P. solanacearum*. Treatment of the lysates with proteinase K and subsequent IET resulted in the patterns of LPS, but none of the other bands were observed (Fig. 2, lanes p). The lipopolysaccharidic character of the patterns was confirmed by silver staining after treatment with proteinase K (Fig. 2D). Results obtained by IET and silver staining agreed for the 33 isolates for which both techniques were used.

Table 4 shows the 98 isolates grouped by their LPS patterns: 15 smooth-type LPS patterns (groups I to XV) and 1 rough-type LPS pattern (group XVI) could be detected. Most of the patterns are shown in Fig. 3. The grouping was based on results of silver staining and IET in terms of the number, intensity, and relative mobility of the electrophoretic bands. LPS profiles were stable in all criteria used for LPS grouping in all replications.

Isolates included in LPS group I reacted with AS-2445, isolates included in group II reacted with AS-536.7.1, and isolates included in group III were recognized by AS-592.4.4. Most of isolates in LPS group VII and some of those in group VI reacted with AS-592.4.4. Other reactions of the isolates of each group with the antisera are shown in Table 4.

Differences in LPS patterns between wrinkled-type colony bacteria and the corresponding smooth-type colony bacteria obtained upon repeated culture (29.1.r, 29.2.r, and 29.3.r, wrinkled; 29.1.l, 29.2.l, and 29.3.l, smooth) were studied, and only changes in band intensity were observed. The profile of the LPS of smooth-type colony bacteria showed decreased intensity for the highest-molecular-weight bands and increased intensity for the lowest-molecular-weight bands compared with those of the wrinkled-type colony bacteria.

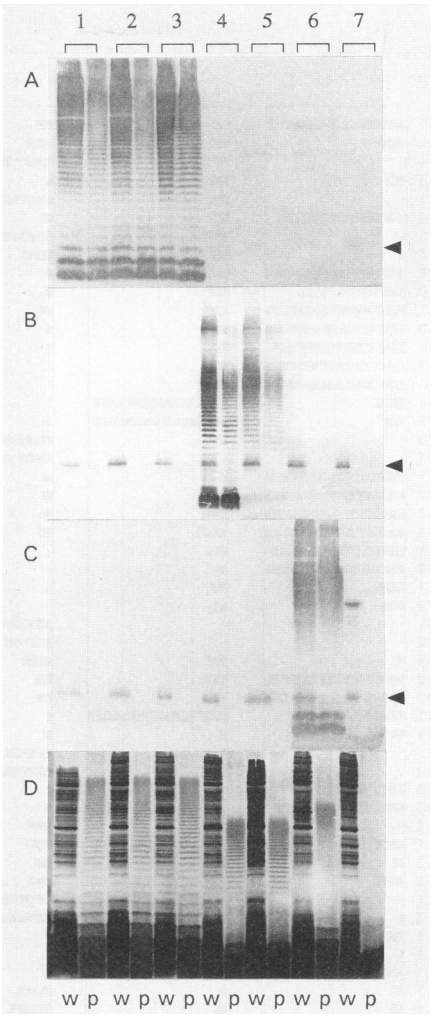

FIG. 2. Electrophoresis and IET by the technique of Towbin et al. (31) with lysates from seven isolates of *Pseudomonas corrugata*. (A) IET with antiserum 2445; (B) IET with antiserum 536.7.1; (C) IET with antiserum 592.4.4; (D) silver-stained gel. Lanes: w, whole-cell lysates; p, samples treated with proteinase K; 1, isolate 2445 (type strain); 2, isolate 313; 3, P. Cor. 1; 4, isolate 536.7.1; 5, isolate 2455; 6, isolate 592.4.4; 7, isolate 83.83.4. Arrowheads indicate one of the protein bands observed in all of the isolates.

DISCUSSION

Despite the widespread occurrence of outbreaks of *P. corrugata*, its biochemical, physiological, and serological features among a large number of isolates have not been previously compared. The aim of this work was to analyze isolates from different geographical origins, providing a wide representation of the *P. corrugata* species.

The isolates of *P. corrugata* studied produced typical tomato pith necrosis, but the length of the internal necrosis showed large variations. Pith necrosis was easily observed when high relative humidity was maintained 2 days after inoculation.

P. corrugata has been described as forming wrinkled colonies and producing yellow pigment on nutrient dextrose

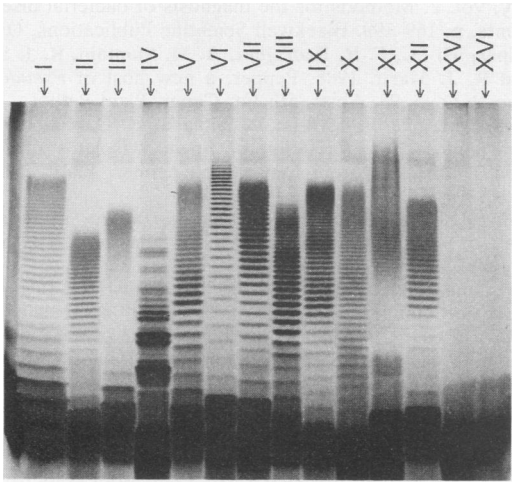

FIG. 3. Different LPS patterns of *P. corrugata* after polyacrylamide gel electrophoresis of whole-cell lysates treated with proteinase K. Numbers correspond to those of the LPS groups in Table 4. The isolates used for each group were as follows: I, 2445; II, 536.7.1; III, 592.4.4; IV, J.375; V, 6; VI, F1; VII, JPc.2; VIII, Pc.12; IX, 2451; X, 8895; XI, 536.1.1; XII, 792; XVI, 83.83.4 and 614.5.3.

agar (27). The isolates studied in this work showed differences in morphology on PYGA, and upon repeated culture, the bacterial colonies sometimes became smooth and the production of pigment faded. The instability of the wrinkled aspect of the colonies has been previously described (23, 27).

Biochemical study showed differences in carbohydrate utilization and exoenzymatic activities. There are discrepancies between results of different authors (25) in lipase, lecithinase, and arginine dihydrolase activities. We also found differences in the ability to hydrolyze gelatin. The morphological change of colonies of *P. corrugata* from the wrinkled to the nonpigmented smooth form was correlated with the inability to hydrolyze gelatin, lecithin, and Tween 80. Bacterial evolution during repeated culturing of *P. corrugata* can modify the results of these tests used for diagnosis of this species.

The presence of some common somatic antigenic determinants has been pointed out for isolates reacting against the same antiserum. Some isolates (C1, 53, 55, 614.5.3, and 2458) reacted against two antisera showing the simultaneous presence of epitopes from isolates 2445 and 536.7.1. Only one of the isolates studied (83.83.4) showed epitopes common with isolates 536.7.1 and 592.4.4. Nevertheless, the results showed high serological variability among *P. corrugata* isolates that confirms the observations of other authors (6, 24). SR calculation seems to be useful for comparing different isolates with a number of antisera, avoiding the problems derived from the use of different microplates during the experiment. The use of serology for routine diagnosis of *P. corrugata* is limited by the large number of serotypes that could be established. LPS is frequently the major antigen from gram-negative bacteria because of its abundance in the bacterial envelope. The high polysaccharide specificity of the O-antigenic chain makes LPS a very important target for antibodies (9). In some cases, bacterial serotyping has been justified as a consequence of bacterial LPS heterogeneity (9, 35). The *P. corrugata* LPS composition is very variable among isolates, as shown by the differences in number, intensity, and mobility of the electrophoretic bands and the specificity against the antisera. This variability involves changes in the monosaccharide composition of the O-antigenic side chain and in the enzymatic specificity of the polymerases and ligases necessary for assembly and transference to lipid A-core molecules (12). Furthermore, differences in electrophoretic migration of the leading band may be due to differences in lipid A-core molecule composition.

The use of LPS to prepare specific antiserum of *P. corrugata* seems to be inadequate because of the high number of LPS types. Other antigens, like the two protein bands detected by the antisera in all of the isolates, are common to other *Pseudomonas* species. The other proteins detected with AS-592.4.4 are present only in some isolates. These results suggest that serology will not be a useful method for routine diagnosis of *P. corrugata* unless specific antigens can be identified.

The available information about LPS composition of phytopathogenic bacteria is limited. Heterogeneity in LPS composition has been described for *Xanthomonas campestris* associated with pathovars (18) and is not associated with *P. syringae* pathovars (35). Great variety in LPS patterns has also been described for strains of *Pseudomonas* plant growth-stimulating bacteria (10). Considering the high number of LPS groups in *P. corrugata*, it would be interesting to know whether the O-antigenic chains are common to other species, as has been reported for *P. cichorii* and *P. solanacearum* (34).

There is no evidence for a correlation between the structural differences observed in LPS of *P. corrugata* isolates and plant-bacterium recognition or virulence. Most of the studied isolates have been obtained from tomato plants, and those from alfalfa and pepper plants do not belong to any special LPS group. No qualitative differences in pathogenicity among isolates of different LPS groups, as described for *P. solanacearum* (3, 13) were observed. Furthermore, in a previous work, inoculation of pepper and tomato plants with isolates from both hosts caused similar pith necrosis (22).

No correlation between LPS groups and geographical origin was observed. Moreover, bacteria belonging to different LPS groups were isolated from the same greenhouse but were not isolated from the same plant.

This is the first comparative study of the characteristics of *P. corrugata* isolated from different origins. Biochemical, physiological, and serological heterogeneity has been observed in the isolates of this ubiquitous bacterium. LPS are determinants of the serological variability observed in this study.

ACKNOWLEDGMENTS

We thank A. M. Gutiérrez-Navarro for helpful discussions during this study and B. Alarcón for assistance with serological techniques. We also thank to L. Gardan, S. Köhn, K. Naumann, E. Griesbach, M. Scortichini, H. Kuwata, K. Oikawa, K. Olsson, P. Persson, J. Vogelsanger, R. Grimm, W. P. Bond, L. L. Black, J. B. Jones, and F. L. Lukezic for kindly providing their isolates. Appreciation is expressed to E. Carbonell for statistical analysis and to A. Marrero and N. Durán-Vila for critical reading of the manuscript.

This work was supported by grants from the Centro de Investigación y Tecnología Agraria, Tenerife, Spain, and the Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain. F.S. was supported by a grant from the Instituto Nacional de Investigaciones Agrarias, Madrid, Spain.

REFERENCES

1. **Alarcón, B., M. M. López, M. Cambra, M. T. Gorris, and J. Guerri.** 1990. Differentiation of *Erwinia carotovora* subsp.

carotovora and *Erwinia carotovora* subsp. *atroseptica* isolated from potato by western blot and subsequent indirect ELISA. J. Appl. Bacteriol. **69:**17–24.

2. **Alarcón, B., M. M. López, M. Cambra, and J. Ortiz.** 1987. Comparative study of *Agrobacterium* biotypes 1, 2, and 3 by electrophoresis and serological methods. J. Appl. Bacteriol. **62:**295–308.
3. **Baker, J. M., J. Neilson, L. Sequeira, and K. G. Keegstra.** 1984. Chemical characterization of the lipopolysaccharide of *Pseudomonas solanacearum*. Appl. Environ. Microbiol. **47:**1096–1100.
4. **Batteiger, B., W. T. Newhall, and R. B. Jones.** 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55:**297–307.
5. **Blake, M. S., A. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1983. A rapid, sensitive method for detection of alkaline phosphatase conjugated anti-antibody on Western blots. Anal. Biochem. **136:**175–179.
6. **Clark, R. G., and D. R. W. Watson.** 1986. New plant disease record in New Zealand: tomato pith necrosis caused by *Pseudomonas corrugata*. N.Z. J. Agric. Res. **29:**105–109.
7. **Cother, E. J.** 1987. A note on the use of Merckoquant test strips for detection of nitrate reduction in plant pathogenic bacteria. Plant Pathol. **36:**374–376.
8. **De Boer, S. H.** 1990. Immunofluorescence for bacteria, p. 295–298. *In* R. Hampton, E. Ball, and S. De Boer (ed.), Serological methods for detection and identification of viral and bacterial plant pathogens. APS Press, St. Paul, Minn.
9. **De Boer, S. H., and M. E. McNaughton.** 1987. Monoclonal antibodies to lipopolysaccharide of *Erwinia carotovora* subsp. *atroseptica* serogroup I. Phytopathology **77:**828–832.
10. **de Weger, L. A., B. Jann, K. Jann, and B. Lugtenberg.** 1987. Lipopolysaccharides of *Pseudomonas* spp. that stimulate plant growth: composition and use for strain identification. J. Bacteriol. **169:**1441–1446.
11. **Fiori, M., C. Carta, and A. Franceschini.** 1983. Il saggio immunoenzimatico (ELISA) per la diagnosi precoce e rapida della necrosi del midollo di pomodoro da *Pseudomonas corrugata* Roberts et Scarlett. Phytopathol. Mediterr. **22:**22–26.
12. **Goldman, R. C., and F. Hunt.** 1990. Mechanism of O-antigen distribution in lipopolysaccharide. J. Bacteriol. **172:**5352–5359.
13. **Hendrick, C., and L. Sequeira.** 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. **48:**94–101.
14. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
15. **Hugh, R., and E. Leifson.** 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol. **66:**24–26.
16. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
17. **Kovaks, N.** 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature (London) **178:**703.
18. **Laakso, T., T. Ojanen, I. M. Helander, R. Karjalainen, and T. K. Korhonen.** 1990. Comparison of outer membrane proteins and lipopolysaccharides of *Xanthomonas campestris* pathovars, p. 149–154. *In* Proceedings of the 7th International Conference on Plant Pathogenic Bacteria. Akadémiai Kiadó, Budapest.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
20. **Lelliott, R. A., and D. E. Stead.** 1987. Methods in plant pathology, vol. 2. Methods for the diagnosis of bacterial diseases of plants, p. 169–199. Blackwell Scientific Publications, Oxford.
21. **López, M. M., F. R. Rodríguez, A. M. Montojo, R. J. Salcedo, and R. J. Martí.** 1988. Pepper, a new host of *Pseudomonas corrugata*, p. 98. Abstr. 5th Int. Congr. Plant Pathol.
22. **López, M. M., F. Siverio, R. Albiach, F. García, and F. R. Rodríguez.** Characterization of Spanish isolates of *Pseudomonas corrugata* from tomato and pepper. Plant Pathol., in press.
23. **Lukezic, F. L.** 1979. *Pseudomonas corrugata*, a pathogen of tomato, isolated from symptomless alfalfa roots. Phytopathology **69:**27–31.
24. **Naumann, K., E. Griesbach, and E. Lattauschke.** 1990. Occurrence and importance of bacterial stem pith necrosis of tomato plants in GDR, p. 473–478. *In* Proceedings of the 7th International Conference on Plant Pathogenic Bacteria. Akadémiai Kiadó, Budapest.
25. **Roberts, W. P., and C. M. Brewster.** 1991. Identification of wheat rhizosphere bacteria inhibitory to root growth. Australas. Plant Pathol. **20:**47–51.
26. **Ryder, M. H., and M. A. Borrett.** 1990. Root colonization by non-fluorescent pseudomonads used for the control of wheat take-all, p. 302–307. *In* C. Keel, B. Koller, and G. Défago (ed.), Plant growth-promoting rhizobacteria—progress and prospects. The 2nd International Workshop on Plant Growth-Promoting Rhizobacteria. IOBC/WPRS Bulletin XIV/8:252–255. Interlaken, Switzerland.
27. **Scarlett, C. M., J. T. Fletcher, P. Roberts, and R. A. Lelliott.** 1978. Tomato pith necrosis by *Pseudomonas corrugata* n. sp. Ann. Appl. Biol. **88:**105–114.
28. **Schaad, N. W.** 1988. Initial identification of common genera, p. 1–15. *In* N. W. Schaad (ed.), Laboratory guide for identification of plant pathogenic bacteria. American Phytopathological Society, St. Paul, Minn.
29. **Scortichini, M.** 1989. Occurrence in soil and primary infections of *Pseudomonas corrugata* Roberts and Scarlett. J. Phytopathol. **125:**33–40.
30. **Thornley, M. J.** 1960. The differentiation of *Pseudomonas* from other gram negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. **23:**37–52.
31. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Nat. Acad. Sci. USA **76:**4350–4354.
32. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
33. **Van Outryve, M. F., M. T. Cerez, M. De Cleene, J. Swings, and T. W. Mew.** Pathogenic pseudomonads associated with sheath rot and grain discoloration of rice *In* Proceedings of the 8th International Conference on Plant Pathogenic Bacteria, in press.
34. **Varbanets, L. D., N. A. Kocharova, Y. A. Knirel, V. A. Muras, N. V. Moskalenko, and O. S. Brovarskaya.** 1991. Lipopolysaccharides of *Pseudomonas solanacearum* and *Pseudomonas cichorii.* Rev. Plant Pathol. **11–12:**900.
35. **Zdordvenko, G. M., N. Y. Gubanova, L. P. Solyanic, A. Yu, L. M. Knirel, and I. Y. Zakarova.** 1991. Composition and structure of lipopolysaccharides from the strains of different pathovars of *Pseudomonas syringae*, p. 391–401. *In* Proceedings of the 4nd Working Group on *Pseudomonas syringae* Pathovars. International Society of Plant Pathology Committee on Phytopathogenic Bacteria and Università di Firenze, Florence, Italy.