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Phenotypic and genomic evidence for the revision of *Pseudomonas corrugata* and proposal of *Pseudomonas mediterranea* sp. nov.

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To re-examine the taxonomic status of *Pseudomonas corrugata*, 27 strains of this species were studied using a polyphasic approach. Numerical analysis of phenotypic data revealed two phena, A (including the *P. corrugata* type strain) and B, which could be clearly differentiated by the assimilation of *meso*-tartrate, 2-ketogluconate and histamine. The mean DNA reassociation values with labelled DNA of *P. corrugata* type strain CFBP 2431^T (phenon A) and strain CFBP 5447^T (phenon B) were high for strains belonging to the same phenon (96·9 and 98·5%, respectively), whereas the DNA relatedness between the two phena was assessed as being close to 70%, which represents the value that is accepted for the definition of a bacterial species. Phena A and B were also differentiated by means of DNA profiles generated by heteroduplex mobility assay of PCR products of 16S rDNA hypervariable region 2, *Hae*III restriction of the amplified internal transcribed spacer, REP- and BOX-PCR profiles, and by PCR with two pairs of specific primers. A comparison of the 16S rRNA sequences of strains CFBP 5447^T and CFBP 5458 from phenon B with the available sequences of *Pseudomonas* species showed that these strains formed a cluster distinct from the *P. corrugata* type strain. Thus, a new species, *Pseudomonas mediterranea*, is proposed for strains of phenon B. The type strain is strain CFBP 5447^T (= ICMP 14184^T); its G+C content is 60·2 mol%.

Keywords: *Pseudomonas corrugata*, *Pseudomonas mediterranea* sp. nov., polyphasic taxonomy, phenotypic clustering, genomic variability

INTRODUCTION

The genus *Pseudomonas* has been thoroughly revised over the last 20 years (Kersters *et al.*, 1996). At present, the genus *Pseudomonas sensu stricto* includes species within rRNA similarity group I (Palleroni, 1984) or the *fluorescens* rRNA branch (De Vos *et al.*, 1985) and belongs to the γ-subclass of the *Proteobacteria* (Kersters *et al.*, 1996). This genus refers to essentially fluorescent species but also includes some non-fluorescent species, of which *Pseudomonas corrugata* is an example. *Pseudomonas corrugata* Roberts and Scarlett 1981 emend. Sutra *et al.* (1997) was first described as being responsible for tomato pith necrosis (Scarlett *et al.*, 1978). It has also been reported to cause a similar disease on chrysanthemum (Fiori, 1992), pepper (Lopez *et al.*, 1994) and geranium (Magyarosy & Buchanan, 1995). Nevertheless, *P. corrugata* is a ubiquitous bacterium and has been isolated from a variety of sources: from soil and from the rhizospheres of different plant species (Lukezic, 1979; Scortichini, 1989; Ryder & Borrett, 1991; Paulitz *et al.*, 1992; Achouak *et al.*, 2000); from rice grains (Van Outryve *et al.*, 1992); from mungbean sprouts (Bennik *et al.*, 1998); and from broccoli heads (Padaga *et al.*,

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Abbreviations: HMA, heteroduplex mobility assay; ITS, internal transcribed spacer; rep-PCR, repeat sequence primed PCR; BOX-PCR, PCR targeting BOXA subunit of *Streptococcus pneumoniae* BOX element; REP-PCR, repetitive extragenic palindromic sequence PCR.

The GenBank accession numbers for the 16S rDNA sequences of strains CFBP 5447^T and CFBP 5458 are AF386080 and AF386081, respectively.

2000). *P. corrugata* does not produce fluorescent pigments, and accumulates medium-chain-length poly(hydroxyalkanoates) (Kessler & Palleroni, 2000; Solaiman *et al*., 2000). Chemotaxonomic studies based on analyses of quinone and fatty acids (Stead, 1992) and on SDS-PAGE of whole-cell proteins (Vancanneyt *et al*., 1996) have placed *P. corrugata* among the fluorescent pseudomonads. Moreover, on the basis of 16S rDNA sequence analysis, Anzai *et al*. (2000) placed *P. corrugata* in the *Pseudomonas fluorescens* group. The emended description of *P. corrugata* based on a polyphasic study indicated that this species appeared to be closely related to three unnamed *Pseudomonas* genomospecies – FP1, FP2 and FP3 (FP, fluorescent pseudomonads) – associated with tomato pith necrosis (Sutra *et al*., 1997).

Evidence of the variability of *P. corrugata* has been provided in different studies in which phenotypic, chemotaxonomic and serological properties and DNA/DNA hybridization data were compared (Siverio *et al*., 1993, 1996; Catara *et al*., 1997; Sutra *et al*., 1997). Three phenotypic groups (1, 2 and 3) were identified among *P. corrugata* strains isolated from Sicily, Italy (Catara *et al*., 1997). Sutra *et al*. (1997) observed that the phenon, which included 87 *P. corrugata* strains from all over the world, was divided into two subphena, 1a and 1b; the mean percentages of DNA reassociation with the *P. corrugata* type strain were 91 % for strains of subphenon 1a and 75 % for strains of subphenon 1b, providing evidence of genomic variability among *P. corrugata* strains. Genomic variability has been also observed in *P. corrugata* by means of enterobacterial repetitive intergenic consensus-PCR and amplified rDNA restriction analysis (Achouak *et al*., 2000) and by random amplified polymorphic DNA (Catara *et al*., 2000). A collection of *P. corrugata* strains analysed by multiplex PCR with two pairs of specific primers produced one of the two possible specific bands, and were consequently assigned to two groups, I and II, respectively (Catara *et al*., 2000). In this study, *P. corrugata* strains from subphena 1a (three strains) and 1b (three strains) of Sutra *et al*. (1997) showed an amplification band of type I and type II, respectively (Catara *et al*., 2000).

In the present study, 27 strains representative of the various groups previously observed within *P. corrugata* (Catara *et al*., 1997; Sutra *et al*., 1997) were investigated using numerical analysis of phenotypic tests, DNA–DNA hybridization, DNA fingerprinting techniques and 16S rDNA sequence analysis. The outcome of this study is the revision of the taxonomic status of *P. corrugata* and the description of a new *Pseudomonas* species for which the name of *Pseudomonas mediterranea* is proposed.

METHODS

Bacterial strains. Twenty-seven *P. corrugata* strains were chosen on the basis of phenotypic data previously obtained by Sutra *et al*. (1997) and Catara *et al*. (1997) (Table 1). They are designated under their CFBP (Collection Française des Bactéries Phytopathogènes, Angers, France) number. Type strains of six major fluorescent oxidase-positive *Pseudomonas* species were also included in this study: *Pseudomonas aeruginosa* CFBP 2466^T, *P. fluorescens* CFBP 2102^T, *Pseudomonas putida* biovar A CFBP 2066^T, *Pseudomonas marginalis* pathovar *marginalis* CFBP 1387^T, *Pseudomonas fuscovaginae* CFBP 2065^T and *Pseudomonas cichorii* CFBP 2101^T. All strains were routinely cultivated on King's B medium.

Numerical analysis of phenotypic data. Twenty conventional biochemical tests were performed as described by Sutra *et al*. (1997). The assimilation of 99 organic substrates was tested using Biotype 100 strips (bioMérieux) as recommended by the manufacturer. Results were read visually after 4 d incubation at 26 °C. The results of these 119 tests were used for a numerical analysis performed as previously described (Sutra *et al*., 1997). A diagnostic coefficient capacity value was determined for each test (Descamps & Véron, 1981).

DNA–DNA hybridization and DNA base composition. Extraction of DNA and DNA–DNA hybridization experiments were performed as reported by Sutra *et al*. (1997). Native DNAs of *P. corrugata* type strain CFBP 2431^T and of strain CFBP 5447^T were labelled *in vitro* by random priming (Feinberg & Vogelstein, 1983) using a Megaprime DNA-labelling system (RPN 1604; Amersham International). DNA–DNA hybridization experiments were repeated two or three times. The G+C content of strain CFBP 5447^T was determined by means of the thermal denaturation temperature (Marmur & Doty, 1962), and were calculated using the equation of Owen & Lapage (1976).

PCR of the ribosomal operon. Three regions, HV1, HV2 and HV3, of the 16S rDNA of *P. corrugata* strains and of *P. aeruginosa* CFBP 2466^T, which contained the hypervariable regions *hv1*, *hv2* and *hv3*, respectively, as defined by Moore *et al*. (1996), were amplified by PCR. The primers were defined from the 16S rDNA sequence of the *P. aeruginosa* type strain (Moore *et al*., 1996). The following primer pairs were used: fD1 (5′-AGAGTTTGATCATGGCTCAG-3′) and rSSU2 (5′-GTAAGAGTCTGGACCGTGTC-3′) for HV1; fSSU1 (5′-AGACACGGTCCAGACTCCTACG-3′) and rSSU1 (5′-ACGCATTTCACCGCTACACAGG-3′) for HV2; and fSSU4 (5′-AGTCCACGCCGTAAACGATGTC-3′) and rSSU3 (5′-TCACCGGCAGCTTCCTTAGAGT-3′) for HV3. The internal transcribed spacer (ITS) between 16S rRNA and 23S rRNA genes was amplified using primers D21 and D22 (Manceau & Horvais, 1997). All PCRs were performed in a 50 µl reaction volume. A 2 µl volume of freeze-and-sow bacterial cells (OD_{600} = 0·5) was added to 48 µl PCR mixture [75 mM Tris/HCl, pH 9·0 (at 25 °C), 20 mM $(NH_4)_2SO_4$, 0·01 % Tween 20, 2·5 mM $MgCl_2$, 0·125 mM (each) dATP, dCTP, dGTP and dTTP (Eurogentec), 25 pmol each primer, and 1 U thermostable DNA polymerase (Goldstar Red; Eurogentec)]. PCRs were performed in a PTC 100 (MJ Research) thermocycler with the following steps: an initial denaturation step at 94 °C for 3 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; and a final extension step at 72 °C for 15 min. PCR products were separated in 1·5 % (w/v) agarose gel (Eurogentec) in TAE buffer (Sambrook *et al*., 1989).

Heteroduplex mobility assay (HMA). For each amplified HV region of the 16S rDNA, heteroduplexes were formed by combining 9 µl of the PCR product of each strain of *P. corrugata*, 9 µl of the PCR product of the corresponding region of *P. aeruginosa* CFBP 2466^T and 2 µl annealing buffer (1 M NaCl, 100 mM Tris, 20 mM EDTA). DNA was

Table 1. Origin of *P. corrugata* strains used in this study

All strains were isolated from sites of pith necrosis on tomato, except strain CFBP 10148 (from pepper) and strain CFBP 5459 (from soil in which infected tomato plants were grown). CFBP, Collection Française des Bactéries Phytopathogènes, Angers, France; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand.

Original phenon	CFBP strain no. (other designation)	Geographic origin
1a*	2431^{T} (ICMP 5819)	England
	10058 (ICMP 8893)	New Zealand
	10146, 10890, 10900, 10904, 10950	Spain
	10938	Switzerland
	10532	France
1b*	10148, 10894, 10961	Spain
	10558	France
1†	5444 (8.1), 5434 (1.4), 5447 (9.1), 5458 (3C)	Italy
2†	5436 (2.1), 5438 (3.1), 5442 (7.6), 5449 (10.3), 5451 (10.8), 5454 (A1), 5456 (D1), 5459 (4.3t)	Italy
3†	12342 (536.7.1), 12343 (614.5.3)	Spain

* Phenon described by Sutra *et al*. (1997).

† Phenon described by Catara *et al*. (1997).

denaturated at 95 °C for 5 min, annealed by rapid cooling in ice and maintained in ice for 1·5 h before analysis. The DNA fragments were separated in 5% (w/v) polyacrylamide gels in TBE buffer (0·044 M Tris, 0·044 M boric acid, 0·001 M EDTA) at 250 V for 2 h (Delwart *et al*., 1993). Gels were stained with ethidium bromide and examined under UV light.

Restriction of ITS. Amplified DNA from the ITS was restricted with two high-frequency cutting enzymes, *Hae*III and *Hinf* I (Eurogentec). A 10 µl volume of PCR product was used and digestion performed with 10 U enzyme per reaction as recommended by the manufacturer. Restricted DNA was analysed by agarose gel electrophoresis as described above.

REP- and BOX-PCR. Strains were analysed by REP-PCR and BOX-PCR (Versalovic *et al*., 1994). PCR conditions were like those described by de Bruijn (1992) and Louws *et al*. (1994). Cell suspensions in sterile water (10^9 c.f.u. ml^{-1}), from 24 h bacterial cultures grown on nutrient glucose agar, were used as templates. Amplification was performed on the Gene Amp System 9700 (PE Applied Biosystems) using 25 µl reaction volumes. PCR products were separated by 1·5% (w/v) agarose gel electrophoresis in TAE buffer (Sambrook *et al*., 1989) at 5 V cm^{-1} over 5 h. A distance matrix based on bands was calculated using the Jaccard coefficient, and a dendrogram was constructed with the unweighted pair group arithmetic average-linkage algorithm method using the NEIGHBOR program of the PHYLIP package (version 3.5c) (J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA). Three analyses were performed for REP bands alone, BOX bands alone and REP and BOX bands together, respectively.

16S rDNA sequencing and phylogenetic analyses. The 16S rDNA genes of strains CFBP 5447^{T} and CFBP 5458 were amplified as described by Achouak *et al*. (1999). The PCR products were purified using the QIAquick PCR purification kit (Qiagen), and then subjected to direct sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the instructions of the manufacturer. DNA sequencing was performed using the previously described primers (Achouak *et al*., 1999). The 16S rDNA sequences were automatically, and then manually, aligned with reference to a database of 35000 already aligned bacterial 16S rDNA sequences. They were then blasted against the current content of the EMBL database (Bacteria division) to check for the presence of newly submitted related sequences. Phylogenetic trees were constructed according to three different methods. BioNJ was performed according to Gascuel (1997), and maximum-likelihood and maximum-parsimony data were obtained from PHYLIP (Phylogeny Inference Package, version 3.573c, distributed by J. Felsenstein, Department of Genetics, UW, Seattle, WA, USA). For the bioNJ analysis, a matrix distance was calculated according to the Kimura two-parameter correction. Bootstraps were done using 500 replications, bioNJ and Kimura two-parameter corrections. The domains used to construct phylogenetic trees were regions of 16S rDNA sequences available for all sequences and excluding positions likely to show homoplasy.

PCR amplification with *P. corrugata*-specific primers. A PCR was performed with type I primers PC5/1 and PC5/2 and type II primers PC1/1 and PC1/2 (Catara *et al*., 2000) on a Gene Amp PCR system 9700 (PE Applied Biosystems). The PCR programme used by Catara *et al*. (2000) was slightly modified as follows: one cycle of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, with a final extension step of 5 min at 72 °C. Amplified products were resolved by electrophoresis at 5 V cm^{-1} in 1·5% (w/v) agarose in TAE buffer (Sambrook *et al*., 1989).

Pathogenicity tests. Pathogenicity tests were performed in the greenhouse with a set of six strains of each of the two groups revealed by our phenotypic results (see Results and Discussion). Four-week-old plants of tomato (*Lycopersicon esculentum* cv. Camone, cv. Cronos, cv. Cemcara), pepper (*Capsicum annuum* cv. Lamuio), eggplant (*Solanum melon-*

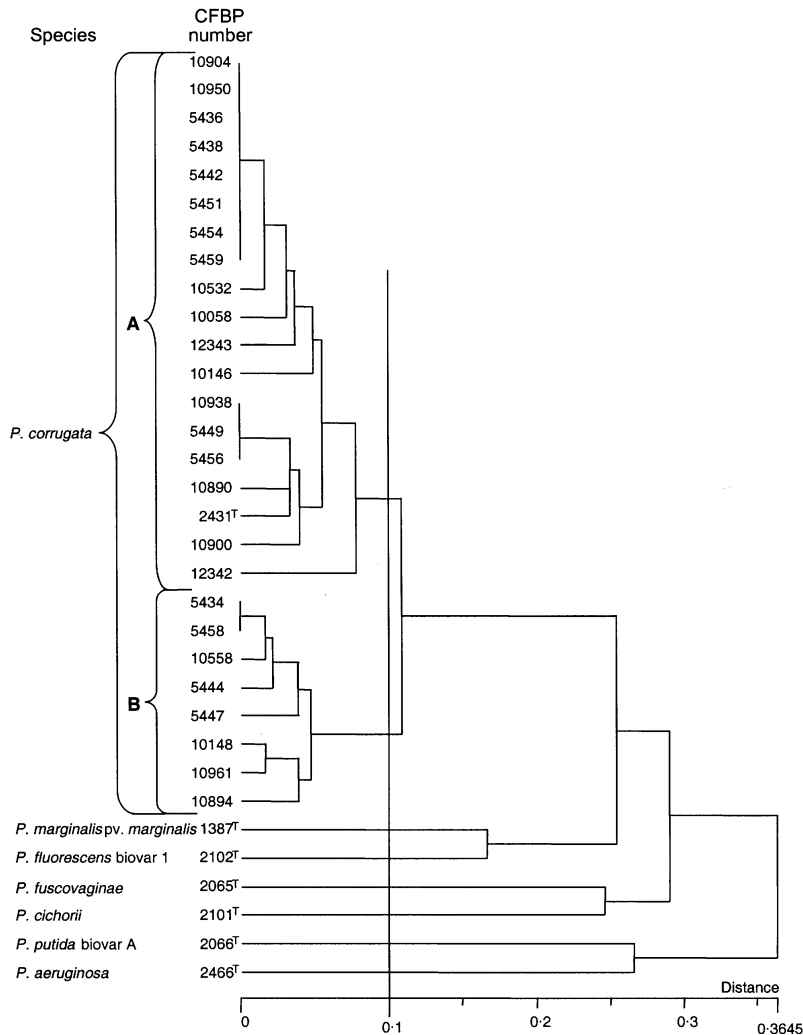

Fig. 1. Dendrogram of phenotypic distances between the 33 strains under study.

gena cv. Gitana), tobacco (*Nicotiana tabacum* cv. Burley), cucumber (*Cucumis sativus* cv. Frontera), watermelon (*Citrullus lanatus* cv. Crimson Sweet) and courgette (*Cucurbita pepo* cv. Romano) grown in trays were inoculated by injecting 50 μl bacterial suspension into the stem at the axil of the first true leaf (five plants of each species or cultivar per bacterial strain). Control plants were inoculated with sterile distilled water. The inoculum consisted of bacterial suspensions in sterile distilled water prepared from a 24-h-old culture and were adjusted to a concentration of 10^8 c.f.u. ml^{-1}. After inoculation, plants were enclosed in polyethylene bags to maintain 100 % relative humidity for 3 days; the bags were then removed until the end of the experiment. Plants were rated for symptoms after 14 days: the stem of each plant was cut longitudinally and the length of pith necrosis measured. Reisolation of bacteria from each plant species tested was performed on nutrient glucose agar.

RESULTS AND DISCUSSION

Numerical taxonomy of phenotypic data

The major biochemical characteristics of the *P. corrugata* strains used in this study were similar to those reported by Sutra *et al.* (1997). A dendrogram displaying distance relationships among the 33 strains under study is presented in Fig. 1. At the distance level of 0·1, two phena and six unclustered strains were observed. Phena A and B contained 19 and eight *P. corrugata* strains, respectively, the *P. corrugata* type strain CFBP 2431^T being included in phenon A. Phena A and B and unclustered strains were clearly differentiated on the basis of phenotypic traits (Table 2). Strains of *P. corrugata* included in phenon B utilized *meso*-tartrate, 2-ketogluconate and histamine, whereas strains of *P. corrugata* belonging to phenon A did not. The phenon A defined in the present study corresponds to subphenon 1a described by Sutra *et al.* (1997) and phena 2 and 3 described by Catara *et al.* (1997), whereas phenon B corresponds to subphenon 1b reported by Sutra *et al.* (1997) and phenon 1 reported by Catara *et al.* (1997).

DNA–DNA hybridization and DNA base composition

The results obtained in this study, together with those previously reported by Sutra *et al.* (1997) using the same experimental conditions, are shown in Table 3.

Table 2. Phenotypic characteristics that differentiate *P. corrugata* phena A and B and fluorescent *Pseudomonas* type strains

Type strains: 1, *P. fluorescens* CFBP 2102^T; 2, *P. fuscovaginae* CFBP 2065^T; 3, *P. cichorii* CFBP 2101^T; 4, *P. putida* CFBP 2066^T; 5, *P. aeruginosa* CFBP 2466^T; 6, *P. marginalis* CFBP 1387^T. +, Positive; −, negative; d, variable (percentage of positive strains in parentheses).

Characteristic (diagnostic coefficient capacity)	Phenon (no. strains)		Type strain					
	A (19)	B (8)	1	2	3	4	5	6
Fluorescence on Kings B medium (0·68)	−	−	+	+	+	+	+	+
Nitrate reduction (0·53)	+	+	−	−	−	+	+	−
Assimilation of:								
meso-Tartrate (0·94)	−	+	+	+	+	+	−	−
2-Ketogluconate (0·94)	−	+	+	−	−	+	+	+
Histamine (0·88)	−	+	−	−	−	+	+	−
DL-Lactate (0·68)	+	+	−	−	−	−	−	−
Mannitol (0·68)	+	+	−	−	−	−	−	−
D-Xylose (0·53)	+	+	+	−	−	−	−	+
Sucrose (0·44)	+	+	−	−	−	−	+	−
D(+)-Trehalose (0·44)	+	+	+	+	−	−	−	+
myo-Inositol (0·44)	+	+	+	−	+	−	−	+
N-Acetylglucosamine (0·44)	+	+	+	−	−	−	+	+
Benzoate (0·44)	−	−	+	−	−	+	+	−
D(−)-Tartrate (0·43)	d (63)	−	−	−	+	−	−	+

Table 3. Results of DNA–DNA hybridization experiments

Values in parentheses are ΔT_m values (°C); NT, not tested.

Unlabelled DNA from:	Hybridization (%) at 70 °C with labelled DNA from:	
	CFBP 2431^T	CFBP 5447^T
Group A strains		
CFBP 2431^T	100	68 (4·2)
CFBP 10146	86	NT
CFBP 5449	102	67 (3·9)
CFBP 5451	92	NT
CFBP 12342	91 (0·9)	NT
CFBP 10058	106*	NT
CFBP 10900	98*	NT
CFBP 10904	98*	NT
Mean±SD	**96·6±6·6**	**67±0·7**
Group B strains		
CFBP 5447	69 (5·6)	100
CFBP 10894	NT	92
CFBP 5434	71 (4·7)	94
CFBP 10558	71	103
CFBP 5458	71	101
CFBP 10148	69 (3·0)*	NT
CFBP 10558	66 (3·0)*	101
Mean±SD	**69·5±2**	**98·5±4·3**

* Values from Sutra *et al.* (1997).

The percentages of reassociation with *P. corrugata* type strain CFBP 2431^T (phenon A) were over 86·0% for the strains of phenon A. They ranged from 66·0 to 71·0% for strains of phenon B, with a mean ΔT_m value of 4·1 °C (range, 3·0–5·6 °C). The percentages of reassociation of strains CFBP 2431^T and CFBP 5449 of phenon A with strain CFBP 5447^T (phenon B) were 68·0 and 67·0%, with ΔT_m values of 4·2 and 3·9 °C, respectively. The percentages of reassociation of strains of phenon B with *P. corrugata* strain CFBP 5447^T (phenon B) were greater than 92·0%. The G+C content of strain CFBP 5447^T was 60·2 mol%.

Thus the DNA–DNA hybridization percentages and ΔT_m values observed for the two phena are close to the borderline applied to the bacterial species, i.e. approximately 70% of DNA–DNA hybridization and ΔT_m values of 5 °C or less (Wayne *et al.*, 1987). However, it has been pointed out that hybridization percentages of around 70% or ΔT_m values of around 5 °C should be carefully interpreted (Grimont, 1988). The presence of phenotypic consistency among strains should be the deciding factor when attempting to delineate a species (Stackebrandt & Goebel, 1994). Moreover, the 70% DNA relatedness values proposed by Wayne *et al.* (1987) should be considered as indicative rather than absolute, and an alternative phylogenetic species concept could delineate a species in a phylogenetic framework as determined by 16S rRNA analyses (Vandamme *et al.*, 1996).

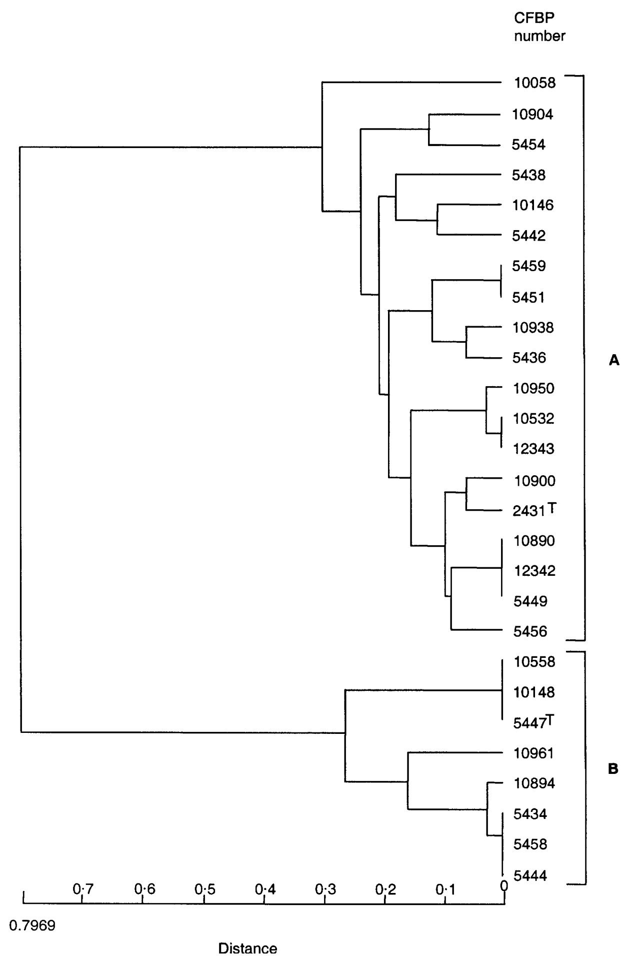

Fig. 2. Dendrogram of distances between the 27 strains of *P. corrugata*, based on total bands obtained by REP- and BOX-PCR.

REP- and BOX-PCR

Complex fingerprints were generated: they consisted of 17–25 REP-PCR bands ranging from 0·5 to 7·0 kb and 15–20 BOX-PCR bands ranging from 0·5 to 5·0 kb. Analysis of distances between strains, using the unweighted pair group arithmetic average-linkage algorithm method, indicated that both the REP and BOX primers were equally effective in delineating two genotypes (A and B). The dendrogram of distances shown in Fig. 2 is based on the analysis of REP and BOX-PCR bands together. The 19 strains of phenon A belong to genotype A and the eight strains of phenon B belong to genotype B. Only eight of the 49 REP-PCR scored bands and five of the 34 BOX-PCR scored bands appeared to co-migrate among the two genotypes. REP- and BOX-PCR fingerprinting profiles were highly similar within each genotype. The numbers of fingerprinting patterns delineated by REP- and BOX-PCR were 15 and 9 for genotype A, and 4 and 3 for genotype B, respectively. The distinct or identical patterns could not be correlated with the geographic origins of isolates.

DNA fingerprints specific to *Xanthomonas campestris* and *Pseudomonas syringae* pathovars have been obtained using rep-PCR techniques (Louws *et al.*, 1994), and a strong correlation has been observed between the grouping of *Xanthomonas* strains based on fingerprints generated by rep-PCR and the genomic species delineated by DNA–DNA hybridization (Rademaker *et al.*, 1997, 2000; Vauterin *et al.*, 2000). These results suggest that these techniques could complement DNA–DNA hybridizations in the definition of bacterial species.

HMA and ITS restriction analysis

Three DNA fragments of about 340 bp (for HV1), 380 bp (for HV2) and 370 bp (for HV3) were amplified from the 16S rDNA of all *P. corrugata* strains and of *P. aeruginosa* type strain CFBP 2466^{T}. These DNA fragments appeared as a single band in agarose gel electrophoresis (data not shown). HMA performed on PCR products from HV1 and HV3 regions of the 16S rDNA generated a unique profile for all of the 27 *P. corrugata* strains tested, whereas HMA performed on PCR products from the HV2 region generated two different profiles (Fig. 3), which coincided with phena

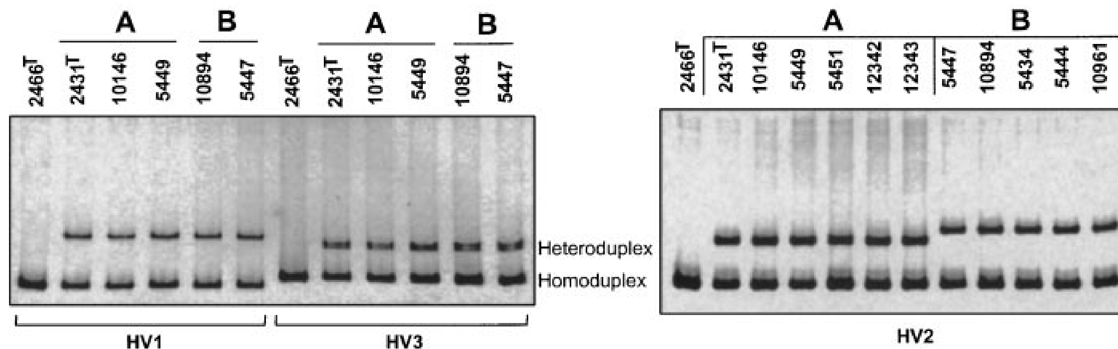

Fig. 3. Examples of HMA profiles of HV1 and HV3 (left) and HV2 (right) regions of the 16S rRNA gene.

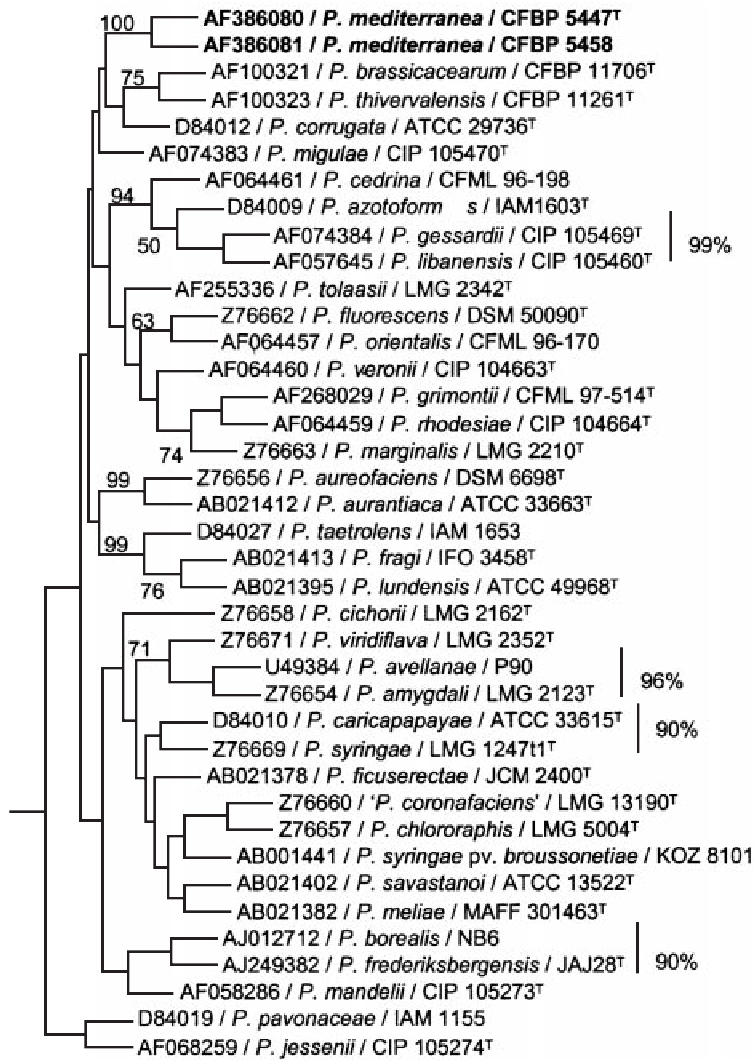

Fig. 4. Unrooted phylogenic tree obtained by 16S rDNA sequence analysis. The topology shown is a restricted subset of a larger analysis including type species of the genus *Pseudomonas*. This tree was obtained using the bioNJ algorithm and 500 bootstrap replications with a Kimura two-parameter correction for the distances. Percentage bootstrap values are indicated only for branches that were also retrieved by maximum parsimony and maximum likelihood ($P < 0{\cdot}01$); these branches should be considered as the only robust clusters identified by this analysis.

A and B described above and revealed sequence differences between them. HMA has been shown to be a powerful technique for screening sequence dissimilarities between annealed strands of DNA (Delwart *et al.*, 1993), and has recently been applied to the assessment of the 16S rDNA similarity of bacterial isolates (Espejo *et al.*, 1998). Approximately 44% of the total 16S rRNA gene sequence variability observed for *Pseudomonas* species occurs in 6% of the gene sequence and is located in the three hypervariable regions *hv1*, *hv2* and *hv3* (Moore *et al.*, 1996). Amplification of the ITS yielded a DNA fragment of about 550 bp. Restriction of the amplified ITS with *Hinf*I generated the same restriction pattern for all of the *P. corrugata* strains tested. In contrast, two different patterns were generated for strains from phena A and B using *Hae*III: pattern 1 consisted of two bands of about 330 and 220 bp, respectively, and pattern 2 consisted of a band of 227 bp and supplementary bands of less than 100 bp. Because the ITS region is highly variable in sequence and size between bacterial organisms, its characterization made it possible to differentiate closely related taxa (Jensen *et al.*, 1993). For example, it has recently been used to differentiate *P. syringae* pathovars or groups of pathovars (Manceau & Horvais, 1997) and *Pseudomonas stutzeri* genomovars (Guasp *et al.*, 2000).

16S rDNA sequence analysis

The 16S rDNAs of strains CFBP 5447^{T} and CFBP 5458 were shown to have 99·2% similarity, and had 98·4 and 98·8% similarity with the 16S rDNA of the *P. corrugata* type strain, respectively. The type strain of *P. corrugata* (phenon A) and strains CFBP 5447^{T} and CFBP 5458 (phenon B) had identical sequences for the *hv1* and *hv3* regions: the *hv1* sequence was identical to that of *Pseudomonas chlororaphis*, and the *hv3* sequence was identical to that of *P. chlororaphis*, *P. marginalis*, *Pseudomonas aureofaciens* and *Pseudomonas tolaasii* (Moore *et al.*, 1996). In contrast, sequence differences were observed between *hv2* regions: the *hv2* region of the *P. corrugata* type strain was identical to that of *P. fluorescens*, whereas the *hv2* region of strains CFBP 5447^{T} and CFBP 5458 was identical to that of *P. marginalis* (Moore *et al.*, 1996).

General large-scale phylogenetic analyses based on 16S rDNA sequences showed that strains CFBP 5447^{T} and CFBP 5458 are members of the γ-branch of the *Proteobacteria*, and that they clustered very robustly within the genus *Pseudomonas sensu stricto* (data not shown). The final analysis (almost the entire 16S rDNA sequences, corresponding to positions 65–1343 of the sequence of strain CFBP 5447^{T}) was effected only with sequences of the genus *Pseudomonas* (55 sequences, mostly of reference strains). The topology presented in Fig. 4, which represents a subset of the total analysis, is that of the bootstrap analysis, as it has been demonstrated that this topology is often better than that of a simple neighbour-joining analysis (Berry & Gascuel, 1996). Strains CFBP 5447^{T} and CFBP 5458 formed a very robust clade, as attested by a bootstrap value of 100 %. Because these strains could not be firmly included in a clade that comprised a single recognized species, our results suggest that these two strains can be equated to a single species. According to our analyses (Fig. 4), this species is included in a larger cluster that comprises the type strains of species such as *P. corrugata*, *Pseudomonas brassicacearum* and *Pseudomonas thivervalensis*.

PCR amplification with *P. corrugata* specific primers

The strains were assigned to group I or group II (*sensu* Catara *et al.*, 2000) based on the sizes of the amplification products. Strains of phenon A were characterized by amplification of the 1100 bp fragment (group I), and strains of phenon B were characterized by amplification of the 600 bp fragment (group II). Thus, each pair of primers, type I and type II, is specific for the identification of strains of the two phena (A and B, respectively).

Pathogenicity tests

All strains tested were able to produce typical symptoms of pith necrosis in tomato, eggplant, pepper and tobacco plants. Brown water-soaked and/or dry pith with cavities which extended above and below the inoculation site was observed in almost all of the inoculated plants. A few of the plants showed a dark-brown hollow lesion around the inoculation site (about 0·5 cm). The extent of the lesion in the pith was variable and ranged from 1 to 8 cm in tomato and from 3 to 11 cm in pepper. Most plants showed discoloration of the vascular system also extending 3–4 cm beyond the pith lesion. When inoculated into cucurbits, almost all *P. corrugata* strains induced yellow to orange discolorations of the pith. Only one strain (CFBP 5444) was clearly less virulent than the other strains, causing yellow discoloration at the inoculation sites on cucumber and watermelon and no symptoms on courgette. No symptoms were observed in control plants. Colonies with characteristics of *P. corrugata* were reisolated from the margins of pith lesions. Randomly selected colonies retrieved from isolation plates were positive in PCRs with *P. corrugata*-specific primers.

P. corrugata has already been shown to induce pith-necrosis symptoms on solanaceous hosts (Siverio *et al.*, 1993; Catara *et al.*, 1997; Sutra *et al.*, 1997). No symptoms on courgette have been previously observed (Sutra *et al.*, 1997). Strains in this study induced lesions whose size varied independently of the strain used, and no statistical difference was observed between the different tomato varieties tested (data not shown). No correlation between pathogenicity or virulence and the distribution of tested strains within the two phena (A and B) was observed. By applying a polyphasic approach combining numerical analysis of phenotypic data, DNA–DNA hybridization, PCR-based DNA typing methods applied to the whole genome (REP- and BOX-PCR) or to selected genetic regions (16S rDNA or ITS), and 16S rDNA sequence analysis, we were able to delineate clearly two closely related, but distinct, taxa within the present *P. corrugata* species. Strains of phenon A, which contain the type strain CFBP 2431^{T}, correspond to true *P. corrugata*. According to our results, strains of phenon B must be assigned to a novel species, for which the name *Pseudomonas mediterranea* is proposed.

Description of *Pseudomonas mediterranea* sp. nov.

Pseudomonas mediterranea (me.di.ter.ra.ne′a. M.L. adj. *mediterranea* mediterranean, referring to the fact that most strains of this species were isolated in Mediterranean countries).

The description of the species is based on results presented in this study and in previous studies (Catara *et al.*, 1997, 2000; Sutra *et al.*, 1997). *P. mediterranea* is a Gram-negative, non spore-forming rod. This species have the same general characteristics as *P. corrugata*. Colonies on YPGA are wrinkled or smooth. Yellow to brown pigments are frequently produced. Motile by means of multiple polar flagella. It is strictly aerobic, non-fluorescent on King's B medium, oxidase-positive, does not produce levan, is not pectolytic, and reduces nitrates into nitrites. Most strains (seven out of eight tested) are arginine dihydrolase-positive after 15 days incubation, and few (two out of eight tested) produce hypersentivity on tobacco leaves. Hydrolysis of Tween 80 and gelatin is variable (seven and six positive strains out of eight tested, respectively). Acid is produced from sucrose and mannitol but not from erythritol and sorbitol. *P. mediterranea* utilizes *N*-acetylglucosamine, D- and L-alanine, 4-aminobutyrate, 5-aminovalerate, D-arabitol, L-arabinose, L-aspartate, *cis*-aconitate, betaine, caprate, caprylate, citrate, ethanolamine, D-fructose, fumarate, D-galactose, D-galacturonate, gluconate, D-glucosamine, D-glucose, D-glucuronate, L-glutamate, glycerol, *p*-hydroxybenzoate, DL-β-hydroxybutyrate, 2-oxoglutarate, DL-lactate, D-lyxose, L-malate, malonate, D-mannitol, D-mannose, mucate, *myo*-inositol, L-proline, propionate, protocatechuate, putrescine, quinate, D-ribose, D-saccharate, sucrose, L-serine, succinate, D-trehalose, *trans*-aconitate, trigonelline, L-tyrosine and D-xylose. In contrast, it does not hydrolyse DNA or aesculin and does not utilize

adonitol, L-arabitol, benzoate, D-cellobiose, *m*-coumarate, dulcitol, erythritol, DL-fucose, gentisate, gentobiose, L-histidine, *m*-hydroxybenzoate, hydroxyquinoline β-glucuronide, itaconate, 5-ketogluconate, lactose, lactulose, maltitol, maltose, maltotriose, D-melezitose, D-melibiose, 1-*O*-methyl α-galactopyranoside, 1-*O*-methyl β-galactopyranoside, 1-*O*-methyl β-D-glucopyranoside, palatinose, phenylacetate, 3-phenylpropionate, D-raffinose, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose, L-tartrate, tricarballylate, L-tryptophan, D-turanose or xylitol. Utilization of the following substrates is variable: D-malate, glutarate and tryptamine. *P. mediterranea* utilizes histamine, 2-ketogluconate and *meso*-tartrate, whereas *P. corrugata* does not. It uses DL-glycerate but not D-tartrate, whereas the assimilation of these two substrates is variable for *P. corrugata*. *P. mediterranea* can be clearly distinguished from *P. corrugata* by 16S rDNA analysis, by means of REP- and BOX-PCR profiles (this study) and by using fingerprints generated by random-primed PCR (random amplified polymorphic DNA) (Catara *et al.*, 2000). Type I primers (PC5/1 and PC5/2) and type II primers (PC1/1 and PC1/2) designed by Catara *et al.* (2000) allow the PCR identification and detection of *P. corrugata* and *P. mediterranea*, respectively. The G+C content of the DNA of the type strain is 60·2 mol% (thermal denaturation method). The type strain is strain CFBP 5447^{T} (= ICMP 14184^{T}). *P. mediterranea* strains were isolated from pith necrosis on tomatoes and peppers, mainly in Italy, Spain and France.

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