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Characteristics of the whole cell fatty acid profiles of *Pseudomonas corrugata*

Fatty acids of P. corrugata

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Abstract

The fatty acid methyl ester (FAME) profiles of eighty strains of *Pseudomonas corrugata* from different geographic origins have been studied. Gas chromatographic profiles were obtained. The use of hexane/methyl-tert butyl ether (MTBE) (1:1) for the extractions improves the yield of hydroxy-FAMEs used to identify *P. corrugata* as compared to hexane alone. The analysis of the extracts with hexane/MTBE showed that many strains do not have the characteristic FAMEs of *P. corrugata* (3-hydroxydodecenoic acid, 3-hydroxytetradecanoic acid, 3-hydroxyhexadecanoic acid and two unknown additional fatty acids). These differences among strains seemed to be related to bacterial dissociation, associated with changes in morphological aspect of the colonies due to subculture. The comparison of profiles of wrinkled and smooth colonies isolated from ten strains confirmed the differences among those in specific FAMEs. Therefore, the FAME profiles are a useful tool for the identification of *P. corrugata* when the bacteria have not been subcultured *in vitro* for a long time. Multivariate analyses of data showed that four clusters can be observed supporting the heterogeneity of the strains of *P. corrugata*.

Abbreviations: FAME – fatty acid methyl ester; MTBE – methyl tert-butyl ether.

Introduction

Pseudomonas corrugata Roberts and Scarlett (1981) is the etiological agent of tomato pith necrosis and is also reported as the responsible for a similar disease in pepper (López et al., 1994) and chrysanthemum (Fiori, 1992). It seems to be a ubiquitous bacterium also isolated from soil (Scortichini, 1990), water (Scarlett et al., 1978), alfalfa symptomless plants (Lukezic, 1979), wheat rhizosphere (Ryder & Borrett, 1990) and rice plants (Van Outryve et al., 1992) for which no rapid identification methods have been described.

P. corrugata forms wrinkled colonies on medium rich in glucose, but this character is not stable since, under repeated culturing, the colonies can become smooth (Lukezic, 1979). There are some biochemical differences between wrinkled and smooth forms of *P. corrugata* (Siverio et al., 1993).

Fatty acid analysis is a useful tool for the identification and classification of bacterial plant pathogens (Miller & Berger, 1985; Stead, 1988; Sasser, 1990; Stead et al., 1992). Most species of the genus *Pseudomonas* can be easily identified (Ikemoto et al., 1978; Oyaizu & Komagata, 1983; Takikawa, 1990; Janse, 1991a; Janse, 1991b; Stead, 1992), even though there are some problems with overlapping profiles, specially to distinguish pathovars (Stead, 1991; Stead, 1992). The whole cell fatty acid profile of *P. corrugata* has been precisely described by Stead (1992) as well as those of the plant pathogenic *Pseudomonas*. This profile has the characteristic peaks of the *Pseudomonas* rRNA group I. Besides, there are significant amounts of

five additional fatty acids (3-hydroxydodecenoic acid, 3-hydroxytetradecanoic acid, 3-hydroxyhexadecanoic acid, and two unknown additional fatty acids) with less than 2% of the whole profile peak area that allows to distinguish *P. corrugata* from other *Pseudomonas* (Stead, 1992). However, the fatty acid profiles of some strains identified as *P. corrugata* in Spain and obtained after extractions with hexane did not have these five characteristic fatty acids (López et al., 1994).

This research undertakes the study of the fatty acid methyl ester (FAME) profiles of *P. corrugata* with a wide collection of strains from different origins. The main differences observed among strains of *P. corrugata* and between wrinkled and smooth forms are described and the usefulness of the FAME profiles in the diagnosis of this bacterium is discussed.

Materials and methods

Bacterial strains. Table 1 lists the 80 strains of *P. corrugata* used in this study, identification of which had been previously confirmed (Siverio et al., 1993). They were routinely grown on King's B medium (King et al., 1954) and peptone yeast glucose agar (PYGA: bactopeptone, 5 g/l; yeast extract, 5 g/l; glucose, 10 g/l; and agar, 20 g/l) and stored at −70 °C in glycerol (25% v:v). The latter medium was used to observe their colony morphology. Wrinkled and smooth colonies of the strains J.374, 903PD, 8894, 1.1.3, 1.2.3, 14.1, 29.1.r, 2445, 2451 and 313, grown on PYGA, were isolated to assess differences between fatty acid profiles of both forms.

Extraction of FAMEs. Bacteria were grown for 24 h at 28 °C on Trypticase Soy Broth Agar (TSBA: Trypticase Soy Broth (BBL # 11768), 30 g/l, plus Bactoagar (Difco # 0140), 15 g/l) and *ca.* 40 mg (wet weight) of cells were harvested. In order to obtain about the same total amount of cells, the total peak area was controlled afterward in the chromatographic profiles. FAMEs were obtained as described by Miller and Berger (1985). The extractions were performed twice with each strain: one with hexane/methyl-tert butyl ether (MTBE) (1:1) and the other with hexane alone. Extractions were performed in batches of 25–30 strains, simultaneously. To evaluate the variation due to culture and chromatographic conditions five strains were repeated four times for each extraction procedure. The extracts were analysed with the Microbial Identification System (MIS, Hewlett-Packard model 5898), controlled with the Microbial Identification System software (MIDI, Microbial ID, Inc., Newark, DE, USA) and using the Aerobe library (the Aerobe TSBA database, version 3.7, January 1993). The MIS includes a gas chromatograph with a 25 m × 0.2 mm 5% methylphenyl silicone fused silica capillary column (H_2 as carrier gas), a flame-ionization detector, an automatic sampler, an integrator and a computer. It

Table 1. Origin of the strains of *P. corrugata*

Location	Source[1]	Strains[2]
France	L. Gardan	83.83.4
Germany	S. Köhn	J.374; J.375; J.609
	K. Naumann and E. Griesbach	V.45; Da.do.2
Italy	M. Scortichini	Pcl.86; 903FS; 903PD; 903T
Japan	H. Kuwata and K. Oikawa	C1; E1; F1; G1
New Zealand	ICMP	7634; 8889; 8890; 8891; 8894; 8895; 8896; 8898; 9303
Spain	IVIA	536.1.1; 536.6.2; 536.7.1; 536.10.2; 542.1.1; 588.2.1; 588.3.1; 614.1; 614.4.1; 614.5.3; 632.2; 632.5; 712.2.a; Ps.Cor.1; T.6; T.7; 1.1.3; 1.1.6; 1.2.3; 2.1; 5.4; 9.2; 12.3; 14.1; 14.4; 14.6; 1113.2; 1113.5; 29.1.r; 29.1.l. 592.4.4; 592.5.4 (*Capsicum annuum*)
Sweden	K. Olsson and P. Persson	53; 54
Switzerland	J. Vogelsanger and R. Grimm	6; 113; 490; 501; 580
United Kingdom	NCPPB	2445; 2447; 2449; 2450; 2451; 2455; 2456; 2457; 2458; 2903
United States	W. P. Bond and L. L. Black	Pc.2; Pc.3; Pc.11
	J. B. Jones	JPc.3; JPc.4
	F. L. Lukezic	792. 299; 313 (*Medicago sativa*)

[1] 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, Britain.
[2] Unless otherwise noted, the host was *Lycopersicon esculentum.*

Table 2. Fatty acids detected in *P. corrugata* and used in this study

Shorthand name	ECL[1]	Systematic name	Common name
10:0	10000	Decanoic acid	Capric acid
10:0 3OH	11423	3-Hydroxydecanoic acid	3-Hydroxycapric acid
12:0	12000	Dodecanoic acid	Lauric acid
Unknown 12486[2]	12486		
12:0 2OH	13178	2-Hydroxydodecanoic acid	2-Hydroxylauric acid
12:1 3OH	13289	3-Hydroxydodecenoic acid	
12:0 3OH	13455	3-Hydroxydodecanoic acid	3-Hydroxylauric acid
Unknown 13961[2]	13961		
14:0	14000	Tetradecanoic acid	Myristic acid
Unknown 14503[2]	14503		
15:0	15000	Pentadecanoic acid	
14:0 3OH	15490	3-Hydroxytetradecanoic acid	3-Hydroxymyristic acid
16:1 *cis* 9	15817	*cis*-9-Hexadecenoic acid	Palmitoleic acid
16:0	16000	Hexadecanoic acid	Palmitic acid
17:0 cyclo	16888	*cis*-9,10-Methylene hexadecanoic acid	
17:0	17000	Heptadecanoic acid	Margaric acid
16:0 3OH	17520	3-Hydroxyhexadecanoic acid	3-Hydroxypalmitic acid
18:1 *cis* 11	17825	*cis*-11-Octadecenoic acid	*cis*-Vaccenic acid
18:0	18000	Octadecanoic acid	Estearic acid
19:0 cyclo C11-C12	18900	*cis*-11,12-Methylene octadecanoic acid	Lactobacillic acid

[1] ECL, equivalent chain length, a liner interpolation peak's retention between two saturated straight-chain fatty acid reference peaks.
[2] The fatty acid peaks with unknown chemical composition were named with their ECL. Mass spectra of the fatty acids unknown 13961 and unknown 14503 indicate that they are branched hydroxy acids containing 13 and 14 carbon atoms, respectively, and for both, the hydroxy group was not in the 2 or 3 position (Stead, 1992).

allows the identification of the fatty acids and their percentage area in the profile, using data of a fatty acid library and a calibration mix. Data were studied by correspondence analysis based on the chi-square distance (Benzecri & Benzecri, 1980). Dendrogram was based on the Ward aggregation criterium (Ward, 1963) which optimized the mean square error of the partition (objective function).

Results/discussion

The twenty-one fatty acids detected in *P. corrugata* and used in this study are shown in Table 2. The reproducibility of profiles observed in the five strains repeated in four batches showed that the major FAMEs with more than 0.5% average peak area had very similar area percent ratios (standard deviation/average relation minimum of 0.02 for 16:0 to a maximum of 0,28 for 17:0 cyclo and an average of 0,11). FAMEs with less than 0.5% peak areas had higher variations and sometimes were not observed in the profiles. There was not relationship between lower total peak areas (less wet weight of cells used for the analysis) and absence of these peaks in the profiles into the ranges of total peak area used. The variation was greater for the cyclopropane acids which tend to increase with the age of the culture (Stead, 1992).

There were some differences in profiles according to the extraction used: the standard hexane/MTBE mixture or hexane alone, this latter simplifying the separation of the phases and being less dangerous to use. Figure 1 shows that in extractions using the hexane/MTBE mixture, FAMEs with less than 1% peak area were detected in more strains and it increased their percent peak areas in the profile. The extractions of other FAMEs, such as 10:0 3OH and 12:0, were also improved. Since we are working with percentages, this increase shows a reduction of some major peaks such as the correspondent to 16:1 *cis* 9 and 18:1 *cis* 11. The differences observed between these two extractors affected the characteristic FAMEs of *P. corrugata* (12:1 3OH, unknown 13961,[1] unknown 14503, 14:0 3OH and 16:0 3OH) that appear in some strains

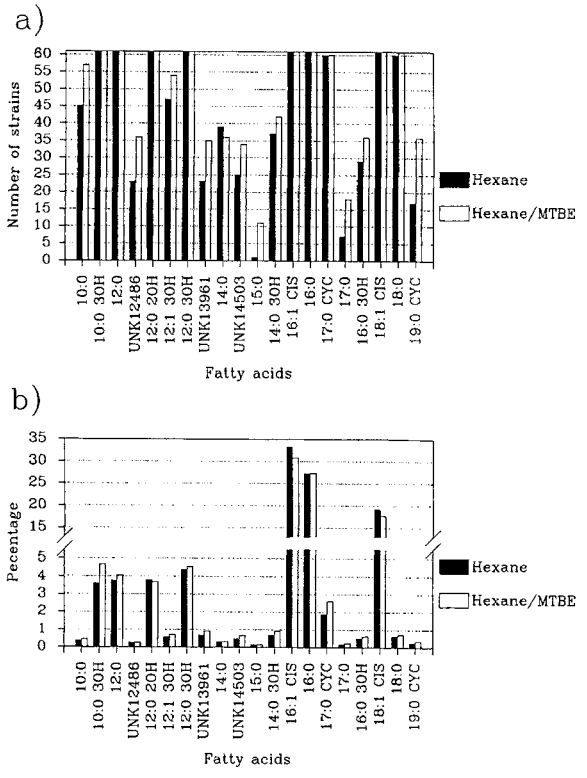

Figure 1. Differences between the extractions using hexane or hexane/methyl-tert butyl ether (MTBE): a) in the number of strains that have each fatty acid methyl esther (FAME) b) in the average percentage of peak area for the FAMEs present.

with hexane/MTBE but not with hexane alone. In the following results the extractions were performed with the hexane/MTBE mixture.

Table 3 summarizes the results observed in the 80 strains analysed, the values previously described for *P. corrugata* (Stead, 1992) and the standard deviations. The main differences observed were the absence in many strains of the FAMEs described as characteristic of *P. corrugata* (12:1 3OH, unknown 13961, unknown 14503, 14:0 3OH and 16:0 3OH) (Stead, 1992) even though the average of the other FAMEs were closely similar in range. These results confirm the differences observed when analysing Spanish isolates of *P. corrugata* (López et al., 1994).

The study of the profiles showed that the FAMEs: unknown 13961, unknown 14503, 14:0 3OH and 16:0 3OH were either all present or all absent in the majority of the profiles (Table 4). These four FAMEs also coincide in most of the profiles with several other FAMEs (hydroxy-FAMEs, 10:0 and 17:0 cyclo) in percentage of peak area over its average and, in less frequency, with unknown 12486 and 19:0 cyclo C11-C12. They

Table 3. Results of fatty acids analysis of 80 strains of *P. corrugata* and comparison with results obtained by Stead (1992)

Fatty acids	x	s	n	x_n	Stead (1992)	
					x	s
10:0	0.42	0.25	75	0.45	0.50	0.40
10:0 3OH	4.68	0.76	80		4.60	0.60
12:0	4.02	0.96	80		3.60	0.30
Unk.12486	0.15	0.14	47	0.25		
12:0 2OH	3.77	0.70	80		3.80	0.20
12:1 3OH	0.66	0.48	72	0.73	1.10	0.50
12:0 3OH	4.58	0.37	80		4.40	0.40
Unk.13961	0.58	0.59	47	0.99	0.80	0.40
14:0	0.17	0.17	43	0.32	tr	
Unk.14503	0.42	0.45	46	0.73	0.50	0.30
15:0	0.02	0.05	14	0.14	tr	
14:0 3OH	0.69	0.62	55	1.00	1.00	0.30
16:1 *cis* 9	30.56	3.01	80		29.10	3.70
16:0	27.36	1.77	80		26.40	1.60
17:0 cyclo	2.64	1.46	77	2.74	5.00	2.60
17:0	0.06	0.10	22	0.22		
16:0 3OH	0.39	0.36	49	0.64	0.50	0.30
18:1 *cis* 11	17.79	1.97	80		17.10	1.40
18:0	0.71	0.33	79	0.72	0.60	0.20
19:0 cyclo C11-C12	0.23	0.22	49	0.37	0.50	0.40

Symbols: x, average of the percentage peak area for all the strains; s, standard deviation; n, number of strains with the fatty acid in their profile; x_n, average of the percentage peak area for the n strains; tr, trace amounts.

Table 4. Coincidences of four characteristic fatty acid methyl esthers of *P. corrugata* in the same profile in 80 strains analysed

Fatty acids				Number of strains
Unknown 13961	Unknown 14503	14:0 3OH	16:0 3OH	
+	+	+	+	46
−	−	−	−	24
+	−	+	+	1
−	−	+	+	1
−	−	+	−	7
−	−	−	+	1

Symbols: +, presence of the fatty acid in the profile; −, absence of the fatty acid in the profile.

seldom appear with 14:0 (only 10 strains) or with 15:0 and 17:0 (data not shown). The comparison of these results with the biochemical characteristics and colony morphology (Siverio et al., 1993) seemed to associate the changes in FAMEs profiles to morphological differences in the colonies (e.g: 35 out of 40 wrinkled strains

Table 5. Observed differences between the FAME profiles of wrinkled colonies and smooth colonies isolated from ten strains

Fatty acids	Wrinkle colonies				Smooth colonies				t-stat
	x	s	n	x_n	x	s	n	x_n	
10:0	0.56	0.22	10		0.20	0.03	10		5.13
10:0 3OH	4.85	0.92	10		3.70	0.20	10		3.86
12:0	3.57	0.46	10		4.93	0.64	10		−5.46
Unk.12486	0.20	0.12	8	0.25	0.08	0.04	7	0.09	3.00
12:0 2OH	4.19	0.58	10		3.14	0.46	10		4.49
12:1 3OH	0.79	0.36	10		0.13	0.08	7	0.18	5.66
12:0 3OH	4.66	0.25	10		4.09	0.10	10		6.69
Unk.13961	0.75	0.54	7	1.06	0.00	0.00	0		4.39
14:0	0.16	0.16	4	0.32	0.31	0.03	10		−2.91
Unk.14503	0.56	0.41	7	0.80	0.00	0.00	0		4.32
15:0	0.02	0.05	1	0.15	0.09	0.06	7	0.13	−2.83
14:0 3OH	0.81	0.58	8	1.01	0.06	0.04	6	0.10	4.08
16:1 *cis* 9	28.61	1.73	10		32.74	1.50	10		−5.70
16:0	27.86	1.87	10		29.20	0.64	10		−2.14
17:0 cyclo	3.56	1.21	10		1.76	0.99	10		3.64
17:0	0.03	0.08	2	0.25	0.18	0.05	10		−5.03
16:0 3OH	0.50	0.29	8	0.62	0.00	0.00	0		5.45
18:1 *cis* 11	17.16	1.29	10		18.56	1.67	10		−2.10
18:0	0.84	0.19	10		0.63	0.09	10		3.16
19:0 cyclo C11-C12	0.34	0.19	8	0.43	0.11	0.09	6	0.18	3.46

Symbols: x, average of the percentage peak area for all the strains; s, standard deviation; n, number of strains with the fatty acid in their profile; x_n, average of the percentage peak area for the n strains; t-stat, t-statistic calculated using non-pooled variance.

displayed all the characteristic FAMEs compared with only 11 out of 40 smooth strains).

The analysis of wrinkled and smooth forms isolated from 10 selected strains of *P. corrugata* revealed they had different profiles, as shown in Table 5. In particular, smooth forms had profiles with less percentage of peak area for hydroxy-FAMEs, 10:0, unknown 12486, unknown 13961, unknown 14503, 17:0 cyclo, 18:0 and 19:0 cyclo C11-C12 than wrinkled ones (Figure 2). These main differences in FAMEs between wrinkled and smooth forms in *P. corrugata* agreed with the main differences observed among the 80 studied strains after multivariate analysis (Figure 3). In this analysis it appeared that strains of *P. corrugata* could be subjectively divided into four clusters: cluster I, grouping mainly wrinkled strains; cluster III, smooth strains; II and IV, both of them. Clusters I and IV contained higher average percentage of peak area than clusters II and III for the FAMEs 12:1 3OH, unknown 13961, unknown 14503, 14:0 3OH and 16:0 3OH. Cyclopropane acid 19:0 cyclo C11-C12 and, especially, 17:0 cyclo are more abundant in clusters I and II. No association with any other of the characteristics of the strains previously studied, such as, lipopolysaccharides and serological reactions (Siverio et al., 1993), was observed. The value of the distance observed among outer strains and the dendrogram (Figure 4) evidence the heterogeneity of the FAME patterns of *P. corrugata*.

The MIDI TSBA database (version 3.7, January 1993) identified 47 out of 80 strains as *P. corrugata* (data not shown). Forty two strains were identified as *P. corrugata* as first option with an average similarity index $(SI)^2$ of 0.81 and five strains as other options with a SI of 0.58. Other strains were classified as *P. savastanoi, P. syringae, P. fluorescens, P. putida, P. cichorii, P. chlororaphis, P. viridiflava* and *P. marginalis*. These species were also other options in the diagnosis of the isolates identified as *P. corrugata*. The MIDI identified as *P. corrugata* 90% of the strains with wrinkled colony morphology and 27.5% of the strains with smooth colony morphology.

According to our results, the use of the FAMEs described as specific to *P. corrugata* or the MIDI

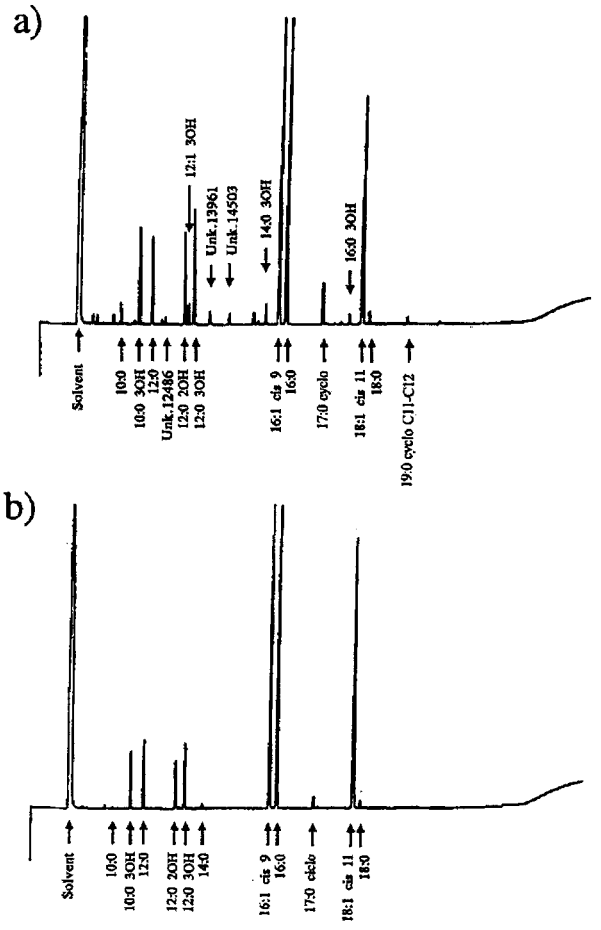

Figure 2. Differences between the fatty acid methyl esther profiles of wrinkled (a) and smooth (b) forms of the 14.1 strain.

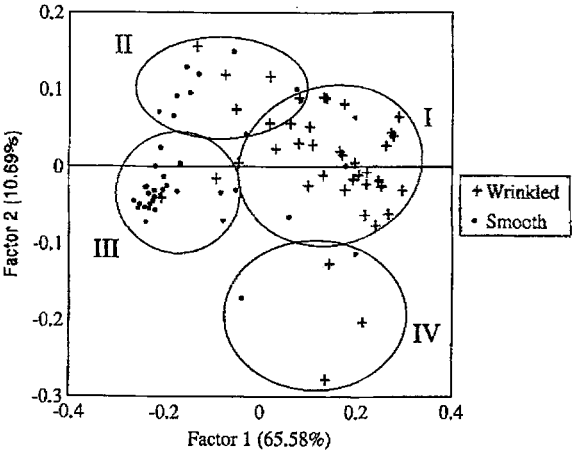

Figure 3. Two dimensional plots of correspondence analysis of 80 strains of *P. corrugata*. Factors 1 and 2 are the first two factors of the correspondence analysis. They are calculated as those linear combinations of original variables that retain the highest percentage of the total variance in addition to be orthogonal to each other. The two factors are responsible for 76% of total variability. Strains of *Pseudomonas* could be subjectively divided into four clusters: cluster I grouping mainly wrinkled strains; cluster III, smooth strains; II and IV, both of them.

system for the identification of this species, may not be as reliable as expected: (i) The main differences between *P. corrugata* and other *Pseudomonas* rRNA group I are five hydroxy-FAMEs with approximately 1% peak areas, that sometimes cannot be detected in the analysis. (ii) Those peaks are not detected in some strains, probably due to bacterial dissociation or polymorphism in culture or to spontaneous variations in *P. corrugata*. (iii) Several characteristic peaks disappear simultaneously from the profiles, probably due to biosynthetic correlation. And, (iv) the profiles without these five FAMEs overlap other *Pseudomonas* rRNA group I (*P. savastanoi, P. syringae, P. fluorescens, P. putida, P. cichorii, P. chlororaphis, P. viridiflava* and *P. marginalis*).

There are few reports in literature concerning FAME profiles and bacterial polymorphism. It has been cited that *P. gladioli* has variations in FAME profiles associated with changes in colony morphology. Rough forms produce profiles with 10:0 and 10:0 3OH, while smooth forms do not (Stead, 1992). Furthermore, 16:1 *cis* 9, 18:1 *cis* 11 and 17:0 cyclo suffer continuous variations across the ranges of values that cannot be arranged in discrete groups (Stead, 1992). Janse (1991a) found that one *P. solanacearum* strain of race 1 (virulent form) changed its atypical fatty acid profile to a typical one in a non-slimy colony type (avirulent form) obtained from it. In *P. corrugata*, changes in colony morphology seem to be related to variations in FAME profiles as well as with the modifications reported in exoenzymatic activities such as lipase, lecithinase and gelatin hydrolysis (Siverio et al., 1993). Differences at the biochemical level associated with morphological changes have also been found in other bacteria such as *P. tolaasii* and *P. gingeri* (Cutri et al., 1984). The variations in FAME profiles of *P. corrugata* were not associated with the electrophoretic profiles of lipopolysaccharides described for this bacterium (Siverio et al., 1993).

In spite of these results, FAME analysis can be employed as a useful technique for the diagnosis of *P. corrugata* considering that bacterial identification is usually done shortly after isolation and bacteria are not yet dissociated. A comparative study of dissociated strains of *P. corrugata* with other *Pseudomonas* having

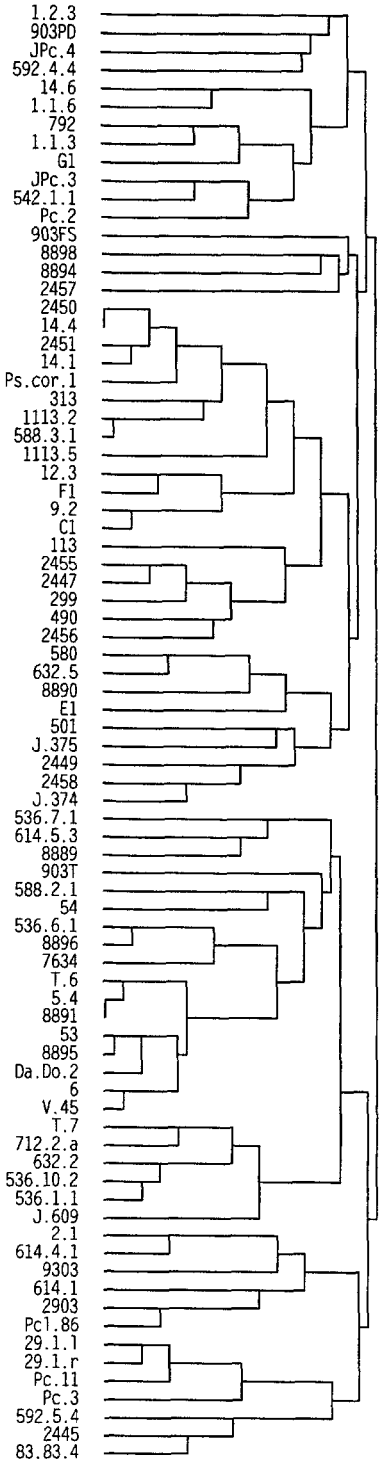

Figure 4. Dendrogram showing the relationships among strains of *P. corrugata* based on fatty acids composition data.

overlapping profiles will surely improve their discrimination, even though it does not seem very promising when considering the results obtained up to date.

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Notes

[1] Fatty acid methyl esthers with unidentified composition will be given with a calculated equivalent chain length number after the word unknown.

[2] Similarity index (SI) is a numerical value, between 0.0 and 1.0, which expresses how closely the fatty acid composition of an unknown isolate compares with the fatty acid composition of the reference strains included in the library. A value between 0.6–1.0 represents an excellent match.

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