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Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov.

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A collection of 42 strains belonging to the five subspecies of *Pectobacterium carotovorum* (subspecies *atrosepticum*, *betavasculorum*, *carotovorum*, *odoriferum* and *wasabiae*) and 11 reference and type strains of biovars of *Pectobacterium chrysanthemi*, *Pectobacterium cacticidum* and *Brenneria paradisiaca* were studied by DNA–DNA hybridization, numerical taxonomy of 120 phenotypic characteristics, serology and new phylogenetic analysis of previously reported sequences from a database of aligned 16S rDNA sequences. The *P. carotovorum* subspecies formed a clade according to neighbour-joining methods, but they formed two paraphyletic clusters according to maximum-likelihood and maximum-parsimony. However, phylogenetic analysis of 16S rDNA sequences alone is not sufficient to justify generic differentiation and therefore, it is proposed to retain the *P. carotovorum* subspecies in the genus *Pectobacterium*. The strains of *P. carotovorum* were distributed in four genomospecies: genomospecies 1, harbouring all strains of subsp. *atrosepticum*, genomospecies 2, including the strains of subsp. *betavasculorum* isolated from sugar beet, sunflower, potato, hyacinth and artichoke, genomospecies 3, clustering all strains of subsp. *wasabiae* isolated from wasabi in Japan, and genomospecies 4, gathering together strains of subsp. *carotovorum* and strains of subsp. *odoriferum*. Four strains of *P. carotovorum* subsp. *carotovorum* remained unclustered. Biochemical criteria, deduced from a numerical taxonomy study of phenotypic characteristics and serological reactions, allowed discrimination of strains belonging to the four genomospecies. Thus, it is proposed that three genomospecies be elevated to species level as *Pectobacterium atrosepticum* sp. nov. (type strain CFBP 1526^T = LMG 2386^T = NCPPB 549^T = ICMP 1526^T), *Pectobacterium betavasculorum* sp. nov. (type strain CFBP 2122^T = LMG 2464^T = NCPPB 2795^T = ICMP 4226^T) and *Pectobacterium wasabiae* sp. nov. (type strain CFBP 3304^T = LMG 8404^T = NCPPB 3701^T = ICMP 9121^T). Only two subspecies are maintained within *P. carotovorum*, subsp. *carotovorum* (type strain CFBP 2046^T = LMG 2404^T = NCPPB 312^T = ICMP 5702^T) and subsp. *odoriferum* (type strain CFBP 1878^T = LMG 5863^T = NCPPB 3839^T = ICMP 11553^T), for which discriminating tests are available.

INTRODUCTION

Pectolytic erwinias responsible for soft rot of plants were classified in three species: *Erwinia cacticida*, *Erwinia carotovora* and *Erwinia chrysanthemi* (Young *et al.*, 1996). The subspecies of *E. carotovora* were historically described as distinct species or subspecies on the basis of pathogenicity and host-plant origin. '*Bacillus carotovorus*' was created for strains isolated from carrot and other vegetables (Jones, 1901). '*Bacillus atrosepticus*' was created for the pathogen causing potato blackleg (van Hall, 1902) and was reduced to a subspecies of *E. carotovora* (Lelliott & Dickey, 1984). This classification, comprising two *E. carotovora* subspecies, was based on the exhaustive study of Dye (1969), who concluded that the many pathogens isolated from various hosts, such as arum, cabbage, carrot, celery, cotton, cucumber, cyclamen, delphinium, hyacinth, maize, potato, sugar cane and tobacco, represented a single species on the basis of their overall common biochemical characteristics. Three other pathogens were subsequently described as subspecies of *E. carotovora*: subsp. *betavasculorum*, responsible for

vascular necrosis of sugar beet (Thomson *et al.*, 1981), subsp. *wasabiae*, responsible for internal discoloration of rhizomes of wasabi (Goto & Matsumoto, 1987), and subsp. *odorifera*, responsible for slimy rot of witloof chicory (Gallois *et al.*, 1992).

For the purposes of identification of *E. carotovora* subspecies, several studies have yielded reliable phenotypic criteria that differentiate two or more subspecies (Graham, 1972; Thomson *et al.*, 1981; Verdonck *et al.*, 1987; de Boer *et al.*, 1987; Goto & Matsumoto, 1987; Alcorn *et al.*, 1991; Gallois *et al.*, 1992). Serological differentiation using the Ouchterlony double-diffusion method was effective for an *E. carotovora* subsp. *atroseptica* diagnosis, but not for *E. carotovora* subsp. *carotovora*, because of the large number of O serogroups (De Boer *et al.*, 1979).

The natural relationships of *Erwinia* species have been studied by analysis of 16S rRNA sequences on the basis of evolutionary trees inferred only by neighbour-joining. Kwon *et al.* (1997) showed that *Erwinia* species formed four phyletic lines. Clade III comprised *E. chrysanthemi* and three subspecies of *E. carotovora* (*carotovora*, *betavasculorum* and *wasabiae*) clustered together. They noticed that the genus *Erwinia* was composed of taxa that displayed considerable heterogeneity and were intermixed with members of other genera belonging to the *Enterobacteriaceae*. Hauben *et al.* (1998) also divided the genus *Erwinia* into three phylogenetic groups. They united the members of clade II, including the five subspecies of *Erwinia carotovora*, *Erwinia cacticida*, *Erwinia chrysanthemi* and *Erwinia cypripedii*, in the genus *Pectobacterium*.

DNA-relatedness studies of soft-rot organisms were first initiated by Brenner *et al.* (1973), who showed that strains of *Pectobacterium carotovorum* and *Pectobacterium chrysanthemi* belonged to distinct DNA homology groups. Of all the soft-rot bacteria, only *E. cacticida* (=*Pectobacterium cacticidum*) (Alcorn *et al.*, 1991) and *E. carotovora* subsp. *odorifera* (=*P. carotovorum* subsp. *odoriferum*) (Gallois *et al.*, 1992) were described on the basis of DNA–DNA hybridization. Recently, using amplified fragment length polymorphism (AFLP) fingerprinting, Avrova *et al.* (2002) defined three clusters corresponding to *P. carotovorum* subspecies: cluster 1 (containing two subclusters, 1a for *P. carotovorum* subsp. *carotovorum* and 1b for *P. carotovorum* subsp. *odoriferum*), cluster 2 (containing two subclusters, 2a for *P. carotovorum* subsp. *atrosepticum* and 2b for *P. carotovorum* subsp. *betavasculorum*) and cluster 3 (for *P. carotovorum* subsp. *wasabiae*).

P. carotovorum is currently divided into five subspecies: *atrosepticum*, *betavasculorum*, *carotovorum*, *odoriferum* and *wasabiae* (Hauben *et al.*, 1998). The purpose of this work was to clarify the taxonomy of the five subspecies by numerical taxonomy, DNA–DNA hybridization, phylogenetic analysis and serology. By using a genotypic approach, we have obtained evidence, presented here, that three subspecies of *P. carotovorum* should be elevated to species level.

METHODS

Bacterial strains. The 53 strains used in this study are listed in Table 1. Forty-two strains of the subspecies of *P. carotovorum* were selected from various host plants and locations all over the world. Ten strains of biovars and pathovars of *P. chrysanthemi*, including the type strain, and the type strain of *P. cacticidum* were included as reference strains.

DNA extraction and DNA–DNA hybridization. Extraction and purification of DNA were performed as described by Brenner *et al.* (1982). Native DNA of *P. carotovorum* subsp. *atrosepticum* CFBP 1526^T, *P. carotovorum* subsp. *betavasculorum* CFBP 2122^T, *P. carotovorum* subsp. *carotovorum* CFBP 2046^T and *P. carotovorum* subsp. *wasabiae* CFBP 3304^T was labelled *in vitro* by nick-translation with tritium-labelled nucleotides (Amersham). The S1 nuclease–trichloroacetic acid method was used for DNA–DNA hybridization (Crosa *et al.*, 1973; Grimont *et al.*, 1980). The thermal stability of reassociated DNA (T_m) was determined by using the method of Crosa *et al.* (1973).

Phenotypic tests. Twenty-two conventional biochemical and physiological tests were performed for all 53 strains as indicated by Sutra *et al.* (2001). All tests were incubated at 28 °C except for growth at 36 and 39 °C in liquid King medium B.

In addition, to study the differentiation of strains by phenotypic tests, a selection of 38 strains, including 28 strains of the five subspecies of *P. carotovorum* and 10 reference strains of *P. chrysanthemi* and *P. cacticidum*, were tested for assimilation of 99 carbon sources, using Biotype 100 strips (bioMérieux). Results were recorded after 4 and 6 days incubation at 28 °C. A total of 121 characteristics were included in a numerical taxonomy analysis for the 38 strains. A distance matrix was calculated using the Jaccard coefficient and a cluster analysis was done using the UPGMA algorithm (Sneath & Sokal, 1973). Discriminatory tests were selected using the diagnostic ability coefficient deduced from the numerical analysis (Descamps & Véron, 1981).

Serology. Antisera were produced in rabbits by injecting formalin-fixed whole bacterial cells. Precipitated antisera were tested by using the Ouchterlony double-diffusion method (Saunier *et al.*, 1996) and gave a single, strong and diffuse band in agar gel, demonstrating a lipopolysaccharide reaction (De Boer *et al.*, 1979).

Phylogenetic analysis. The 16S rDNA sequences of *P. carotovorum* strains and related sequences were selected from a database of 40 000 previously aligned bacterial 16S rDNA sequences. Selection of sequences was based on previous phylogenetic analyses of the entire database and BLAST searches against the latest release of the European Bioinformatics Institute database. Phylogenetic trees were constructed using three different methods: neighbour joining (bioNJ), maximum likelihood (ML) and maximum parsimony (MP). For the NJ analysis, distance matrices were calculated using Kimura's two-parameter correction. bioNJ was applied according to Gascuel (1997), ML and MP were from the PHYLIP package (version 3.573c). For the final tree, 80 sequences of strains closely related to *P. carotovorum* were retained. Because of their close relationships, no evident homoplasy was detected, and almost the entire sequences corresponding to positions 52–1399 of sequence U80197 of *P. carotovorum* subsp. *carotovorum* were used for that analysis. Phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996). When several sequences were available for a type species, all sequences were included (they often differed by a few nucleotides).

Table 1. Strains of *Pectobacterium* used in this study

Abbreviations: CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; NCPPB, National Collection of Plant-pathogenic Bacteria, Sand Hutton, York, UK; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Ghent, Belgium; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand. –, Not known. Biovars (bv.) and pathovars (pv.) of *P. chrysanthemi* are indicated.

Strain	Other designation	Host	Geographical origin	Year of isolation
P. carotovorum* subsp. *atrosepticum				
CFBP 511	Prunier 149-2	Potato	France	1964
CFBP 1330	Pérombelon A21	Potato	UK	–
CFBP 1453	Barzic TO30	Tomato	France	1973
CFBP 1525	Kelman SR8	Potato	USA	1969
CFBP 1526^T	NCPPB 549^T	Potato	UK	1957
CFBP 5836	Jouan 87-7	Potato	France	1987
CFBP 5957	Samson EII 219	Potato	France	1976
CFBP 5958	Samson EII 234	Potato	France	1976
CFBP 5959	Samson EII 316	Potato	USA	1977
P. carotovorum* subsp. *betavasculorum				
CFBP 1520	Fucikowsky 17	Sunflower	Mexico	–
CFBP 2122^T	NCPPB 2795^T	Sugar beet	USA	1971
CFBP 3291	LMG 2398	Potato	Romania	1962
CFBP 3292	LMG 2461	Sugar beet	USA	1974
CFBP 3294	LMG 6681	Sugar beet	USA	–
CFBP 3295	LMG 6682	Sugar beet	USA	–
CFBP 5838	Sf 142-2	Artichoke	La Réunion	1986
P. carotovorum* subsp. *carotovorum				
CFBP 797	NCPPB 550	Tobacco	USA	1951
CFBP 798	NCPPB 2042	Carrot	USA	–
CFBP 1336	Pérombelon C21	Potato	UK	–
CFBP 1350	Mazzucchi 340	Squash	Italy	–
CFBP 1458	Dickey 197	Chrysanthemum	USA	1971
CFBP 1489	Bonnet 118	Chrysanthemum	France	1971
CFBP 1535	NCPPB 552	Maize	Israel	1958
CFBP 2046^T	NCPPB 312^T	Potato	Denmark	1952
CFBP 2136	Sailly 26	Potato	France	1976
CFBP 2137	Sailly 96	Potato	France	1976
CFBP 2138	Sailly 7	Potato	France	1976
CFBP 2139	Sailly 15	Potato	France	1976
CFBP 2140	Samson R59	Potato	France	1976
CFBP 2141	Sailly 872	Potato	France	1978
CFBP 5832	Ngwira CM1	Cabbage	Malawi	1986
CFBP 5833	Ngwira PM2	Potato	Malawi	1986
CFBP 5835*	Jouan 88-29	Potato	France	1988
CFBP 5960	Jouan 87-25	Potato	France	1987
P. carotovorum* subsp. *odoriferum				
CFBP 1878^T	Samson 1177-11^T	Chicory	France	1979
CFBP 2599	Vogelsanger 582	Chicory	Switzerland	1985
CFBP 3296†	LMG 6688	Hyacinth	The Netherlands	–
P. carotovorum* subsp. *wasabiae				
CFBP 3304^T	Goto SR 91^T	Wasabi	Japan	1985
CFBP 3305	Goto SR 36	Wasabi	Japan	1985
CFBP 3306	Goto SR 92	Wasabi	Japan	1985
CFBP 3307	Goto SR 93	Wasabi	Japan	1985
CFBP 3308	Goto SR 94	Wasabi	Japan	1985
P. chrysanthemi				
CFBP 1200 (pv. *dianthicola*; bv. 1)	NCPPB 453	Carnation	UK	1956
CFBP 1270 (pv. *parthenii*; bv. 6)	NCPPB 516	*Parthenium* sp.	Denmark	1957

Table 1. cont.

Strain	Other designation	Host	Geographical origin	Year of isolation
CFBP 1447 (bv. 8)	NCPPB 2546	Maize	India	1969
CFBP 1451 (pv. *paradisiaca*; bv. 4)‡	Granada 142	Plantain	Colombia	1972
CFBP 3477 (pv. *paradisiaca*; bv. 4)‡	ICMP 2349	Plantain	–	–
CFBP 1805 (bv. 9)	Dinesen EKII	Kalanchöe	Denmark	1977
CFBP 2015 (bv. 7)	Samson 314-1	Potato	France	1975
CFBP 2048^T (pv. *chrysanthemi*; bv. 5)	NCPPB 402^T	Chrysanthemum	USA	1956
CFBP 2051 (pv. *dieffenbachiae*; bv. 2)	NCPPB 2976	*Dieffenbachia* sp.	USA	1957
CFBP 2052 (pv. *zeae*; bv. 3)	Kelman SR58	Maize	USA	1970
P. cacticidum				
CFBP 3216^T	Alcorn 67^T	Cactus	USA	–

*Received as *P. carotovorum* subsp. *atrosepticum*.

†Received as *P. carotovorum* subsp. *betavasculorum*.

‡*P. chrysanthemi* bv. 4 (pv. *paradisiaca*) has been reclassified as *Brenneria paradisiaca*, with the type strain CFBP 3477^T.

RESULTS

DNA–DNA hybridization

The results of DNA–DNA reassociation are shown in Table 2. In all, four hybridization groups were delineated. DNA hybridization group 1 included the nine strains of *P. carotovorum* subsp. *atrosepticum*, which demonstrated 89–100 % relatedness to the type strain, CFBP 1526^T (mean, 95·6 %; SD, 3·4 %). These strains constituted genomospecies 1. Strains of the other groups were 36–55 % related to CFBP 1526^T.

DNA hybridization group 2 included the seven strains of *P. carotovorum* subsp. *betavasculorum*, which were 85–100 % (97·4 ± 5·6 %) related to the type strain, CFBP 2122^T. These strains constituted genomospecies 2. Strains of the other groups were 15–63 % related to strain CFBP 2122^T.

DNA hybridization group 3 included the five strains of *P. carotovorum* subsp. *wasabiae*, which were 100 % related to the type strain, CFBP 3304^T. These strains constituted genomospecies 3. Strains of the three other groups were 48–64 % related to CFBP 3304^T, with ΔT_m values ranging from 6·3 to 7·8 °C.

DNA hybridization group 4 included 14 of the 18 strains of *P. carotovorum* subsp. *carotovorum*, which were 62–100 % (76·8 ± 8·5 %) related to the type strain, CFBP 2046^T, with ΔT_m values ranging from 3·1 to 4·9 °C, and two strains of *P. carotovorum* subsp. *odoriferum* (one of which, CFBP 3296, was misidentified, having been received as *P. carotovorum* subsp. *betavasculorum*) that were 70–75 % (72·5 ± 3·5 %) related to strain CFBP 2046^T, with a ΔT_m value of 3·7 °C. These strains corresponded to genomospecies 4. The strains of the other unrelated groups were 42–62 % related to strain CFBP 2046^T, with ΔT_m values ranging from 7·5 to 9·0 °C.

The four remaining strains of *P. carotovorum* subsp. *carotovorum* did not fit with any other genomospecies described above, since they were 46–60 % related to *P. carotovorum* subsp. *carotovorum* CFBP 2046^T (55 ± 6·6 %), with ΔT_m values ranging from 6·2 to 7·3 °C.

The type strain, CFBP 2048^T, and seven reference strains of the biovars of *P. chrysanthemi* and the type strain of *P. cacticidum* were not closely related (7–30 %) to the type strains of the four *P. carotovorum* subspecies.

Phenotypic tests

The use of 22 biochemical and physiological tests confirmed the identity of the bacteria (Gallois *et al.*, 1992) and the repartition in their respective genomic groups, with the exception of two strains: CFBP 5835, received as *P. carotovorum* subsp. *atrosepticum*, which was identified as *P. carotovorum* subsp. *carotovorum*; and CFBP 3296, received as *P. carotovorum* subsp. *betavasculorum*, which was reclassified as *P. carotovorum* subsp. *odoriferum*.

The dendrogram of phenotypic distances among the 38 strains is shown in Fig. 1. At a distance of 0·37, the strains were clustered in three groups: *P. carotovorum*, *P. chrysanthemi* and *P. cacticidum*. The criteria that distinguished these three groups were L(+)-arabinose, malonate, growth at 39 °C, indole, D(+)-malate, D(+)-trehalose and lecithinase. At a distance of 0·15, seven phenons and eight unclustered strains were observed.

Phenon 1 corresponded to 12 strains of *P. carotovorum* subsp. *carotovorum* isolated from various host plants: potato, tobacco, carrot, squash, chrysanthemum, maize and cabbage. Phenon 2 corresponded to five strains of *P. carotovorum* subsp. *atrosepticum* isolated from potato and tomato. Three strains of *P. carotovorum* subsp. *odoriferum* (including the misidentified strain CFBP 3296 isolated from hyacinth) were clustered in phenon 3. The three strains of *P. carotovorum* subsp. *wasabiae* were clustered in phenon 4. For strains of *P. carotovorum* subsp. *betavasculorum*, the three strains isolated from sugar beet and the two strains

Table 2. Levels of DNA relatedness among *Pectobacterium* strains

ΔT_m values (in °C) are shown in parentheses. NT, Not tested.

Source of unlabelled DNA	Relative binding (%) with labelled DNA from strain:			
	1	2	3	4
Genomospecies 1 (*P. carotovorum* subsp. *atrosepticum*)				
CFBP 511	92	NT	NT	56
CFBP 1330	94	59	52	57 (7·7)
CFBP 1453	89	NT	NT	60
CFBP 1525	96	55	58	61 (9·0)
1. CFBP 1526^T	100	47	64 (6·3)	52
CFBP 5836	96	NT	NT	56
CFBP 5957	99	NT	NT	59
CFBP 5958	97	NT	NT	55
CFBP 5959	97	63	58	62 (8·5)
Mean ± SD	95·6 ± 3·4	56 ± 6·8	58 ± 4·9	57·2 ± 3·2
Genomospecies 2 (*P. carotovorum* subsp. *betavasculorum*)				
CFBP 1520	NT	97	NT	55
2. CFBP 2122^T	53	100	50 (7·5)	52 (7·5)
CFBP 3291	NT	100	NT	47
CFBP 3292	NT	100	NT	48
CFBP 3294	51	100	52 (7·5)	53
CFBP 3295	50	85	48	52
CFBP 5838	NT	100	NT	57
Mean ± SD	51·3 ± 1·5	97·4 ± 5·6	50 ± 2	52 ± 3·6
Genomospecies 3 (*P. carotovorum* subsp. *wasabiae*)				
3. CFBP 3304^T	47	47	100	55 (8·9)
CFBP 3305	49	49	100 (0)	42
CFBP 3306	NT	NT	100 (0)	56
CFBP 3307	NT	NT	100 (0)	59
CFBP 3308	NT	NT	100 (0)	60
Mean ± SD	48 ± 1·4	48 ± 1·4	–	54·4 ± 7·2
Genomospecies 4 (*P. carotovorum* subsp. *carotovorum*)				
CFBP 798	NT	NT	NT	72
CFBP 1350	41	46	57	71 (4·9)
CFBP 1458	44	48	50	62 (5·0)
CFBP 1489	48	NT	NT	74 (4·1)
4. CFBP 2046^T	46	53	57 (7·7)	100
CFBP 2136	NT	NT	NT	77 (4·1)
CFBP 2137	NT	NT	NT	83
CFBP 2138	NT	NT	NT	75 (3·3)
CFBP 2139	NT	NT	NT	78 (3·1)
CFBP 2140	55	NT	NT	74 (3·7)
CFBP 2141	NT	NT	NT	77 (4·0)
CFBP 5832	49	54	58 (7·8)	72 (4·4)
CFBP 5833	NT	NT	NT	76 (5·6)
CFBP 5960	NT	51	57	84 (4·6)
Mean ± SD	47·2 ± 4·8	52·7 ± 3·8	57·6 ± 0·9	76·8 ± 8·5
Phenon 3 (*P. carotovorum* subsp. *odoriferum*)				
CFBP 1878^T	45	47	49	70
CFBP 3296	NT	56	NT	75 (3·7)
Mean ± SD	–	51·5 ± 6·4	–	72·5 ± 3·5
Ungrouped strains (*P. carotovorum* subsp. *carotovorum*)				
CFBP 797	42	NT	NT	60 (6·2)
CFBP 1336	36	15	51	46 (7·3)
CFBP 1535	44	NT	NT	60 (6·2)

Table 2. cont.

Source of unlabelled DNA	Relative binding (%) with labelled DNA from strain:			
	1	2	3	4
CFBP 5835	43	NT	NT	54
Mean ± SD	41·3 ± 3·6	–	–	55 ± 6·6
P. chrysanthemi				
CFBP 1200 (bv. 1)	NT	NT	NT	12
CFBP 1270 (bv. 6)	NT	NT	NT	15
CFBP 1447 (bv. 8)	NT	NT	NT	11
CFBP 1451 (bv. 4)	NT	NT	NT	13
CFBP 1805 (bv. 9)	NT	NT	NT	13
CFBP 2015 (bv. 7)	NT	NT	NT	14
CFBP 2048^T (bv. 5)	NT	NT	10	10
CFBP 2052 (bv. 3)	7	7	NT	10
P. cacticidum				
CFBP 3216^T	21	24	20	30

isolated from sunflower and artichoke were respectively clustered in phenons 5 and 6. However, the latter five strains were only clustered in a unique phenon at a distance of 0·24, which suggested phenotypic variability among these five strains, probably related to the host origins of the strains: sugar beet on the one hand and various host plants on the other.

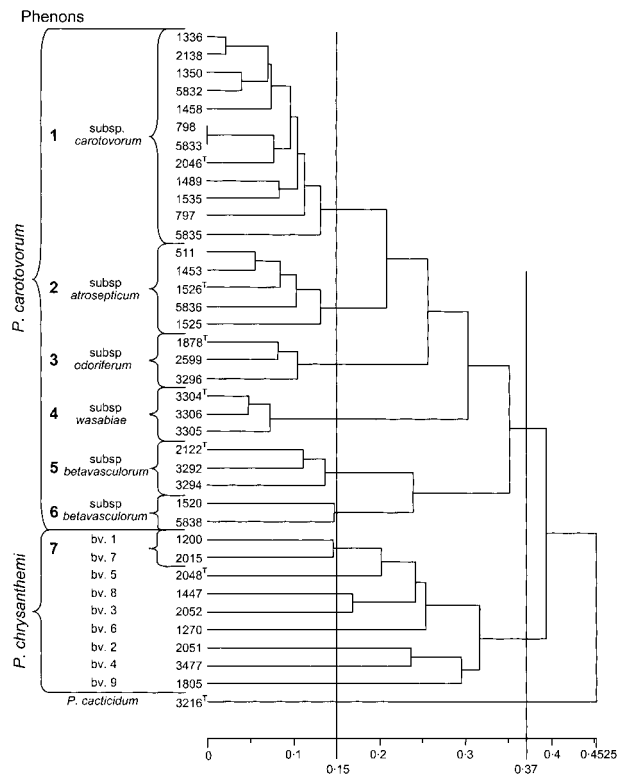

Fig. 1. Dendrogram of phenotypic characteristics of the 38 strains based on the UPGMA algorithm. Distance = 1 − Jaccard coefficient.

Reference strains of the different biovars of *P. chrysanthemi* (two strains of bv. 4, CFBP 1451 and 3477^T, are now named *Brenneria paradisiaca*) and *P. cacticidum* were clustered either in phenon 7 (CFBP 1200 and 2015) or as isolated strains.

The five subspecies of *P. carotovorum* constituted rather homogeneous phenotypic groups, except the aforementioned five strains of *P. carotovorum* subsp. *betavasculorum*. Phenotypic characteristics that differentiate the seven phenons and the reference strains of *P. chrysanthemi* were deduced from the diagnostic ability coefficient given by numerical taxonomy analysis (Table 3). Two to eight tests allowed phenons to be distinguished from one another.

Serology

All strains of genomospecies 1 and phenon 2 (*P. carotovorum* subsp. *atrosepticum*) reacted with the two antisera 102 and 160, except for CFBP 511, which remained rough and untypable (Table 4). All strains of genomospecies 2 and phenons 5 and 6 (*P. carotovorum* subsp. *betavasculorum*) reacted with antiserum 413. The five strains of genomospecies 3 (*P. carotovorum* subsp. *wasabiae*) reacted with antiserum 411. The remaining strains of *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *carotovorum* showed wide serological diversity, since each antiserum only recognized one or two of the strains (Table 4). The three aforementioned subspecies of *P. carotovorum* constitute distinct but uniform serogroups. The correspondence with the serogroups of De Boer *et al.* (1979, 1987) is based upon control reactions of the serological references.

Phylogenetic analysis

The tree in Fig. 2 is a robust subset of a larger tree of 80 sequences. The topology shown is that of the bootstrap tree, as it has been demonstrated that this topology is often better than that of a simple NJ or MP analysis (Berry & Gascuel, 1996).

Table 3. Determinative tests for *P. carotovorum* subspecies

Phenons are indicated as: 1, *P. carotovorum* subsp. *carotovorum*; 2, *P. carotovorum* subsp. *atrosepticum*; 3, *P. carotovorum* subsp. *odoriferum*; 4, *P. carotovorum* subsp. *wasabiae*; 5, *P. carotovorum* subsp. *betavasculorum*. Numbers of strains tested are indicated. Tests are scored as: −, 90–100 % of strains negative; +, 90–100 % of strains positive; numbers indicate percentages of strains that tested positive. DAC, Diagnostic ability coefficient.

Test	DAC	Phenon 1 ($n=13$)	Phenon 2 ($n=6$)	Phenon 3 ($n=3$)	Phenon 4 ($n=3$)	Phenon 5	
						3 strains	2 strains
Palatinose	0·93	−	+	+	−	+	+
1-*O*-Methyl α-D-glucopyranoside	0·93	−	+	+	−	+	+
Production of reducing substances from sucrose	0·93	−	+	+	−	+	+
Lactose	0·82	+	+	+	−	67	50
Inulin	0·77	−	−	−	−	+	+
meso-Tartrate	0·75	−	−	−	+	−	−
α-D(+)-Melibiose	0·75	+	+	+	−	−	50
Gelatinase	0·71	+	67	+	+	−	−
D-(+)-Cellobiose	0·67	+	+	+	+	−	50
D(−)-Tartrate	0·61	−	−	+	−	−	−
Raffinose	0·59	+	+	+	−	+	+
D-Glucuronate	0·59	69	+	−	+	−	+
Growth at 36 °C	0·54	+	17	+	−	+	+
Citrate (Simmons')	0·50	+	83	33	+	−	−
L-Glutamate	0·44	+	33	+	−	67	+
Sorbitol	0·38	−	−	+	−	−	−
D(+)-Arabitol	0·38	−	−	+	−	−	−

DISCUSSION

The aim of this study was to scrutinize the taxonomy of the subspecies of *P. carotovorum*. A representative collection of 53 strains belonging to the five subspecies of *P. carotovorum* was selected and examined for DNA relatedness, phenotypic features and serological properties.

By DNA–DNA hybridization, four genomospecies were delineated. DNA relatedness within genomospecies was 62–100 %, with ΔT_m values ranging from 0 to 5·6 °C. Heterologous DNA relatedness among all genomospecies was 41–63 %, with ΔT_m values of 6·2–9·0 °C. Thus, genomospecies 1–4 correspond to the phylogenetic definition of bacterial species of Wayne *et al.* (1987), taking account of both DNA–DNA relatedness of approximately 70 % or more and ΔT_m values of 5 °C or less. Fourteen of 18 strains previously identified as *P. carotovorum*. subsp. *carotovorum* were grouped in genomospecies 4, including the type strain (CFBP 2046^T). We consider these strains as representing genuine *P. carotovorum*, which we have better circumscribed by DNA–DNA hybridization. We demonstrated genomic heterogeneity among the strains of *P. carotovorum* subsp. *carotovorum*, since four strains identified as *P. carotovorum* subsp. *carotovorum* by classical phenotypic tests remained ungrouped by DNA–DNA hybridization. Further studies on additional strains would be necessary in order to establish the taxonomic position of these unclustered strains. The strains of *P. carotovorum* subsp. *odoriferum* were normally grouped among *P. carotovorum*, and its taxonomic status will remain unchanged (Gallois *et al.*, 1992).

Reports on the genetic diversity of *P. carotovorum* have mainly concerned the subspecies *atrosepticum* and *carotovorum*, because of economic losses of potato crops due to soft rots. Several DNA probes were found to be specific for *P. carotovorum* subsp. *atrosepticum* (Darrasse *et al.*, 1994a; Ward & De Boer, 1994) and PCR primers were proposed for the detection of the same subspecies on potatoes (De Boer & Ward, 1995; Frechon *et al.*, 1998). *P. carotovorum* subsp. *atrosepticum* was also consistently considered rather uniform in terms of PCR-RFLP fingerprints using a *pel* gene (Darrasse *et al.*, 1994b; Helias *et al.*, 1998) and comparison of the *recA* gene (Waleron *et al.*, 2001). By contrast, *P. carotovorum* subsp. *carotovorum* showed far greater diversity: almost 20 RFLP groups were described in the two gene PCR-RFLP studies. Using AFLP fingerprinting, Avrova *et al.* (2002) confirmed the subdivision of *P. carotovorum* into five subspecies, but they did not draw taxonomic conclusions from their results. These reports are consistent with our results, placing *P. carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *betavasculorum* and *P. carotovorum* subsp. *wasabiae* into individual species, and indicate extreme diversity within *P. carotovorum* subsp. *carotovorum*.

Our results allowed the strains clustered in genomospecies 1 (*P. carotovorum* subsp. *atrosepticum*), 2 (*P. carotovorum*

Table 4. Ouchterlony reactions of *Pectobacterium* strains with eight antisera

The strain against which each antiserum is directed (CFBP accession no.) is indicated. The corresponding O serogroups of De Boer *et al.* (1979, 1987) are indicated in parentheses.

CFBP strain no.	Antiserum (target strain)					
	102 (1453), 160 (1526^T)	**413 (2122^T)**	**411 (3304^T)**	**97 (2136)**	**33 (798), 99 (2138)**	**112 (1878)**
Genomospecies 1, phenon 2 (*P. carotovorum* subsp. *atrosepticum*)						
1330, 1453, 1525, 1526^T, 5836, 5957, 5958, 5959	+ (I)	−	−	−	−	−
Genomospecies 2, phenons 5 and 6 (*P. carotovorum* subsp. *betavasculorum*)						
1520, 2121, 2122^T, 3291, 3292, 3293, 3294, 3295, 5838	−	+ (XXXV)	−	−	−	−
Genomospecies 3, phenon 4 (*P. carotovorum* subsp. *wasabiae*)						
3304^T, 3305, 3306, 3307, 3308	−	−	+	−	−	−
Genomospecies 4, phenon 1 (*P. carotovorum* subsp. *carotovorum*)						
1350, 1489, 2046^T, 2139, 2140, 2141, 5832, 5833, 5960	−	−	−	−	−	−
2136, 2137	−	−	−	+	−	−
798, 2138	−	−	−	−	+ (XXXIV)	−
1458	−	−	−	−	−	+ (XVII)
Phenon 3 (*P. carotovorum* subsp. *odoriferum*)						
1878^T	−	−	−	−	−	+
2599, 3296	−	−	−	−	−	−
Ungrouped strains, phenon 1 (*P. carotovorum* subsp. *carotovorum*)						
797, 1336, 1535, 5835	−	−	−	−	−	−
P. chrysanthemi						
1200, 1270, 1447, 1451, 1805, 2015, 2048^T, 2051, 2052, 3477	−	−	−	−	−	−
P. cacticidum						
3216^T	−	−	−	−	−	−

subsp. *betavasculorum*) and 3 (*P. carotovorum* subsp. *wasabiae*) to be readily identified on the basis of biochemical tests and serological reactions. We did not find tests that distinguished *P. carotovorum* subsp. *carotovorum* strains of genomospecies 4 from the four genomically unclustered strains of this subspecies. *P. carotovorum* subsp. *carotovorum* strains demonstrated genomic heterogeneity that was not correlated to phenotypic properties. Similar discrepancies have already been observed for other groups of bacteria, e.g. among strains of *Pseudomonas* isolated from soil or the rhizosphere (Achouak *et al.*, 2000; Bossis *et al.*, 2000).

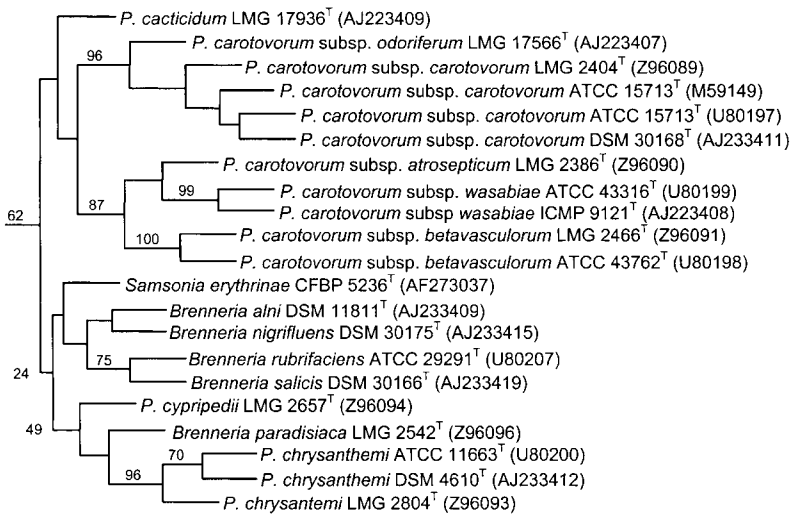

Fig. 2. Rooted tree representing a subset of a larger phylogenetic analysis of 16S rDNA sequences that included closely related outgroups (such as members of *Serratia*, *Enterobacter*, *Erwinia* and *Pantoea*) that were used to root the tree shown. This tree is the result of an NJ bootstrap analysis (1000 replications). Bootstrap percentages are indicated only for branches that were retrieved also by MP and ML. No distance bar is presented because (i) distances are corrected, and (ii) this is a bootstrap tree.

Among the 17 differentiating tests selected on the basis of numerical taxonomy by diagnostic ability coefficient, 12 were previously reported tests, confirmed in their ability to identify the subspecies of *P. carotovorum* (Dye, 1969; Graham, 1972; Thomson *et al.*, 1981; Verdonck *et al.*, 1987; De Boer *et al.*, 1987; Goto & Matsumoto, 1987; Alcorn *et al.*, 1991; Gallois *et al.*, 1992). Of the five other tests, palatinose was already known but is too expensive to be used routinely. *meso*-Tartaric acid assimilation seemed specific for phenon 4 strains, D-(−)-tartaric acid for phenon 3 strains, absence of D-glucuronic acid assimilation for strains of phenons 3 and 5 and L-glutamic acid assimilation testing constantly negative for phenon 4 strains. These five tests represent new criteria useful for the identification of *P. carotovorum* subspecies.

The serological heterogeneity of *P. carotovorum* subsp. *carotovorum* was pointed out previously by De Boer *et al.* (1987), who described 37 O serogroups for this subspecies. In contrast, serology has long been considered useful for recognition of *P. carotovorum* subsp. *atrosepticum* (Graham, 1963; Vruggink & Maas-Geesteranus, 1975). Murray *et al.* (1990) demonstrated that three *P. carotovorum* subsp. *betavasculorum* strains isolated from sugar beet belonged to De Boer's serogroup XXXV. We confirmed this result with eight strains of the same subspecies isolated from three different host plants. Unless contraindicated by examination of a larger collection of strains, particularly of *P. carotovorum* subsp. *wasabiae*, we conclude that antisera can be useful for rapid identification of the three subspecies *atrosepticum*, *betavasculorum* and *wasabiae*.

The phylogenetic analysis revealed that not all subspecies of *P. carotovorum* were grouped in a single, robust clade identified by all methods. This was not a definitive conclusion, but a strong indication that the different subspecies of *P. carotovorum* could indeed belong to different species; the DNA–DNA hybridization data presented above confirmed this speculation. Detailed scrutiny of the results given by each phylogenetic method showed that all *P. carotovorum* subspecies formed a clade according to the NJ method (32 % bootstrap), while they formed two paraphyletic clusters according to MP and ML methods; these clusters corresponded to the two branches identified by NJ. Such a result is very often observed in phylogenetic analyses when the numbers of derived characters are rather small compared with the numbers of sequences analysed. Therefore, a phylogenetic analysis of 16S rDNA sequences alone is insufficient to determine whether all of these species can be placed in a single genus or not. DNA–DNA hybridization values around 50 % and strong phenotypic similarities are then decisive. A similar conclusion can be reached for *P. cacticidum*, which can be included (NJ, 34 % bootstrap) or excluded from the genus (paraphyletic: MP and ML). By contrast, *P. chrysanthemi* and *P. cypripedii* should be excluded from the genus (all methods) according to 16S rDNA phylogenetic analyses.

Using only NJ for inferring evolutionary trees, Hauben *et al.* (1998) grouped the five subspecies of *E. carotovora* in a unique genus, *Pectobacterium*. The results presented above using two other methods (ML and MP) showed that the subspecies of *P. carotovorum* formed two paraphyletic clusters. Phylogenetic analysis of 16S rDNA sequences alone is insufficient to split the *P. carotovorum* subspecies into two genera. Therefore, we propose that these five subspecies of *P. carotovorum* be maintained in the genus *Pectobacterium* first proposed by Waldee (1945). The taxonomic status of *P. cacticidum*, *P. chrysanthemi* and *P. cypripedii* should be revised later.

The taxonomy of soft-rot erwinias has evolved and has been debated over the last four decades. The taxonomic status of *P. carotovorum* was, successively, species (Burkholder, 1957), variety (Dye, 1969) and species again divided into three subspecies, *carotovorum*, *atrosepticum* and *betavasculorum* (Lelliott & Dickey, 1984). Except for *P. carotovorum* subsp. *odoriferum*, these taxonomic proposals were based on biochemical and physiological characteristics only.

Although the species and subspecies of *Pectobacterium* have usually been named according to the host from which each was first isolated, their expanded host range means that pathogenicity is not a determinative test for these taxa. For instance, *P. carotovorum* subsp. *betavasculorum* has been isolated from hosts other than sugar beet (sunflower, potato and artichoke) and *P. carotovorum* subsp. *odoriferum* has been isolated from witloof chicory and hyacinth (this study revealed the true identity of the hyacinth strain) as well as from celery, leek (Gallois *et al.*, 1992) and sugar beet (R. Samson, unpublished).

Our results on DNA–DNA hybridization and phenotypic characteristics and serological reactions are in accordance with the bacterial definition of species of Wayne *et al.* (1987). We consider that the three subspecies of *P. carotovorum* subsp. *atrosepticum*, *betavasculorum* and *wasabiae* should be elevated to species level. We therefore propose the names *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. for these taxa.

P. carotovorum subsp. *carotovorum* and subsp. *odoriferum* share some characteristics with the three novel species described below that differentiate them from *P. chrysanthemi* and *P. cacticidum*: they do not possess lecithinase or arginine dihydrolase, they grow in the presence of 5 % NaCl, they assimilate D-mannitol, sucrose, trehalose, L(+)-arabinose, α-L-rhamnose, D-saccharate, mucate and L-serine and they do not assimilate malonate, L(+)-tartrate, *cis*-aconitate or 4-aminobutyrate. There is no growth at 39°C and no indole production.

Description of *Pectobacterium atrosepticum* (van Hall 1902) Hauben *et al.* 1999 sp. nov.

Basonym: *Pectobacterium carotovorum* subsp. *atrosepticum* (van Hall 1902) Hauben *et al.* 1999.

Does not grow at 36 °C, produces reducing compounds from sucrose, produces acid from α-methylglucoside,

lactose, melibiose, cellobiose and raffinose, does not produce acid from inulin, sorbitol or D(+)-arabitol, assimilates palatinose, lactulose, L-alanine, 1-*O*-methyl β-galactopyranoside and D-glucuronate and does not assimilate *meso*-tartrate, D(−)-tartrate or L-alanine. Causes blackleg of potato and soft rot of potato tubers during storage, and is casually isolated from tomato (Barzic *et al.*, 1976). The G+C content of the DNA ranges from 51·3 to 53·1 mol% (Hauben *et al.*, 1998). The type strain is CFBP 1526^T (=LMG 2386^T =NCPPB 549^T =ICMP 1526^T).

Description of *Pectobacterium betavasculorum* (Thomson *et al.* 1981) Hauben *et al.* 1999 sp. nov.

Basonym: *Pectobacterium carotovorum* subsp. *betavasculorum* (Thomson *et al.* 1981) Hauben *et al.* 1999.

Grows at 36 °C, produces reducing compounds from sucrose, does not possess gelatinase, produces acid from methyl α-glucoside, inulin, lactose and raffinose, does not produce acid from sorbitol or D(+)-arabitol, assimilates palatinose and L-alanine and does not assimilate *meso*-tartrate, D(−)-tartrate or citrate. Causes root vascular necroses of sugar beet and is casually isolated from sunflower, artichoke and potato. The G+C content of the DNA ranges from 54·1 to 54·6 mol% (Hauben *et al.*, 1998). The type strain is CFBP 2122^T (=LMG 2464^T =NCPPB 2795^T =ICMP 4226^T).

Emended description of *Pectobacterium carotovorum* (Jones 1901) Waldee 1945

The description is based on the results of our phenotypic study (Table 3) and those of Hauben *et al.* (1998). Strains grow at 36 °C and possess gelatinase. Strains utilize lactose, α-D(+)-melibiose, D(+)-cellobiose, raffinose and L-glutamate and do not utilize inulin or *meso*-tartrate. The type strain is CFBP 2046^T (=LMG 2404^T =NCPPB 312^T =ICMP 5702^T).

Emended description of *Pectobacterium carotovorum* subsp. *carotovorum* (Jones 1901) Hauben *et al.* 1999

Grows at 36 °C, does not produce reducing compounds from sucrose, possesses gelatinase, produces acid from lactose, melibiose, raffinose and cellobiose, does not produce acid from methyl α-glucoside, inulin, sorbitol or D(+)-arabitol, assimilates 1-*O*-methyl α-galactopyranoside, 1-*O*-methyl β-galactopyranoside and L-glutamate and does not assimilate palatinose, *meso*-tartrate or L-alanine. Strains cause soft rot in a wide range of host plants. The G+C content of the DNA of the type strain is 52·1 mol% (Starr & Mandel, 1969). The type strain is CFBP 2046^T (=LMG 2404^T =NCPPB 312^T =ICMP 5702^T).

Emended description of *Pectobacterium carotovorum* subsp. *odoriferum* (Gallois *et al.* 1992) Hauben *et al.* 1999

The description is given by Gallois *et al.* (1992), and some additional tests are listed in Table 3. Produces volatile flavouring compounds. Strains cause soft rot of witloof chicory during forcing and decay of other plants such as leek, celery and hyacinth. The type strain is CFBP 1878^T (=NCPPB 3839^T =ICMP 11533^T).

Description of *Pectobacterium wasabiae* (Goto & Matsumoto 1987) Hauben *et al.* 1999 sp. nov.

Basonym: *Pectobacterium carotovorum* subsp. *wasabiae* (Goto & Matsumoto 1987) Hauben *et al.* 1999.

Does not grow at 36 °C, does not produce reducing compounds from sucrose, possesses gelatinase, does not produce acid from methyl α-glucoside, inulin, lactose, melibiose, raffinose, sorbitol or D(+)-arabitol, assimilates *meso*-tartrate and D-glucuronate and does not assimilate palatinose, 1-*O*-methyl α-galactopyranoside, lactulose, L-alanine, 1-*O*-methyl β-galactopyranoside or L-glutamate. Causes discoloured rhizomes and fibrous rot of Japanese horseradish (*Eutrema wasabi*). The G+C content of the DNA ranges from 51·4 to 51·7 mol% (Hauben *et al.*, 1998). The type strain is CFBP 3304^T (=LMG 8404^T =NCPPB 3701^T =ICMP 9121^T).

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