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Short Communications

Evaluation of the API 50CH and API ZYM systems for rapid characterization of *Clavibacter michiganensis* subsp. *sepedonicus*, causal agent of potato ring rot

José Luis Palomo[1,*], María M. López[2], Pablo García-Benavides[1], Encarna Velázquez[3] and Eustoquio Martínez-Molina[3]
[1]*Centro Regional de Diagnóstico, Junta de Castilla y León, Apdo. 61, 37080, Salamanca, Spain;* [2]*Instituto Valenciano de Investigaciones Agrarias, Ctra. Moncada-Náquera Km. 4,5, 46113, Moncada, Valencia, Spain;* [3]*Departamento de Microbiología y Genética. Lab. 209. Edificio Departamental, Universidad de Salamanca, Campus Unamuno, 37007, Salamanca, Spain *Author for Correspondence: Fax: +34-923363149; E-mail: jlpg@usal.es*

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Abstract

API 50CH and API ZYM systems were used to characterize fifty-three strains of *Clavibacter michiganensis* subsp. *sepedonicus* from different geographic locations and several reference strains of the same and different species, including other potato pathogens. *Clavibacter michiganensis* subsp. *sepedonicus* strains displayed a high level of homogeneity, both in carbohydrate utilization and in enzymatic activity. Using API 50CH and API ZYM it was possible to differentiate *C. michiganensis* subsp. *sepedonicus* strains from the remaining taxa analysed in this study, which included representative strains of the other subspecies of *C. michiganensis* as well as other bacterial pathogens affecting potatoes. Therefore, these systems could be used as an effective method to characterize *C. michiganensis* subsp. *sepedonicus*. Such a procedure would constitute an alternative system to the conventional nutritional and physiological identification tests currently included in the official methods employed in the European Union to detect and identify this bacterium. The results obtained with the API systems agreed with the current taxonomic classification of *C. michiganensis*, clearly separating *sepedonicus* from the other subspecies belonging to this species.

Clavibacter michiganensis subsp. *sepedonicus*, causal agent of potato ring rot, is a quarantine organism in the European Union (EU), A2 list of Directive 2000/29 (Anon., 2000) and causes a serious potato disease. The official methodology used for analysis is presently regulated by Directive 93/85/EEC (Anon., 1993). The detection of this bacterium in latent infection of tubers is especially complex because tissue extracts must usually be inoculated into eggplant to facilitate its isolation. The isolates must be identified by serology, bioassay and a set of conventional nutritional and physiological tests (Jansen and Van Vaerenbergh, 1987). Since the whole procedure is time consuming, different detection and identification methods based on molecular techniques have been proposed to speed up the process (De Boer et al., 1995; Li et al., 1997; Louws et al., 1998; Wullings et al., 1998; Pastrik and Rainey, 1999; Pastrik, 2000; Palomo et al., 2000; Smith et al., 2001; Beckhoven et al., 2002; Rivas et al., 2002; Fessehaie et al., 2003). Moreover, the European Union has supported a project (DIAGPRO) for the standardization and validation of a protocol to detect and diagnose this quarantine bacterium (available in www.csl.gov.uk/science/organ/ph/diagpro). As a result, a new Directive has been drawn up (publication pending), including the

same nutritional and enzymatic identification tests as in the previous version, together with serological and molecular tests. Such tests should be standardized to obtain equivalent results between laboratories and the ideal procedure would use commercial systems, facilitating the work of diagnostic laboratories.

Clavibacter michiganensis subsp. *sepedonicus* is a Gram-positive coryneform bacterium, strictly aerobic, requiring nutritionally rich media on which it grows slowly (from 5 to 30 days). *Clavibacter* species and subspecies have been identified by means of conventional physiological and biochemical tests or by fatty acid analysis, but these methods are not applicable to large-scale routine testing. Harris-Baldwin and Gudmestad (1996) developed a rapid identification method for phytopathogenic coryneform bacteria based on the utilization of 95 carbon sources using the Biolog automated identification system. However, the results obtained with *Clavibacter* strains were highly variable and their accurate identification varied from 27% to 77% depending on the subspecies. Moreover, the Biolog system is an expensive method for bacterial identification, and not available in most diagnostic laboratories. By contrast, various API systems are accessible to the majority of laboratories. API 50CH contains a high number of carbohydrates, some of which are recommended for identification of *C. michiganensis* subsp. *sepedonicus* in the official analysis methodology, such as glycerol, rhamnose, salicin, lactose, starch and aesculin. The use of API 50CH strips to characterize *C. michiganensis* subsp. *sepedonicus* has not previously been reported. The API ZYM strips enable 20 exoenzymes to be detected and have previously been used in studies of coryneform bacteria, in which some strains of *C. michiganensis* subsp. *sepedonicus* were included (Vantomme et al., 1987; De Bruyne et al., 1992).

The aim of this work was to evaluate the accuracy of these two systems for characterizing *C. michiganensis* subsp. *sepedonicus*. In order to achieve this goal we included isolates of this subspecies from different geographic origins as well as representative strains from other *C. michiganensis* subspecies, several taxonomically related species and bacteria pathogenic on potato.

In this study we used 53 strains of *C. michiganensis* subsp. *sepedonicus* isolated from *Solanum tuberosum* in different geographic locations; 26 of the strains were isolated in Spain since 1994 and 27 strains were from other countries (see Table 1). Reference strains of other bacterial species used in this study are also listed in Table 1. Strains were conserved by cryopreservation at −80 °C, and cultivated in YPGA (yeast extract 5 g l^{-1}, bacto peptone 5 g l^{-1}, glucose 10 g l^{-1}, agar 15 g l^{-1}).

The API 50CH system (bioMérieux, Marcy-l'Etoile, France) includes 50 dehydrated carbon sources in microtubes and standard basal media designed for optimal growth of different bacterial species. However, none of these media proved suitable for the growth of *C. michiganensis* subsp. *sepedonicus* so it was necessary to design a specific medium suitable for the growth of this species. After checking several media (data not shown), we selected YMA as basal medium (Vincent, 1970), without mannitol and with bromothymol blue as pH indicator. In this medium *C. michiganensis* subsp. *sepedonicus* grew well when glucose or mannitol was added, whereas no colour changes were observed in the absence of a carbon source. Measuring turbidity can be used to evaluate the growth of many bacteria in the API 50CH microtubes, but in the case of *C. michiganensis* subsp. *sepedonicus* it was not possible to detect turbidity changes due to its slow growth. Therefore, it was necessary to add a pH indicator to the medium to visualize pH changes. The composition of the modified YMA was as follows: Yeast extract 2.0 g l^{-1}, K_2HPO_4 0.25 g l^{-1}, KH_2PO_4 0.25 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g l^{-1}, $MnSO_4 \cdot H_2O$ 0.15 g l^{-1}, NaCl 0.05 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 0.005 g l^{-1}, bromothymol blue 0.05 g l^{-1}. To prepare the inoculum the strains were plated in NBY medium prepared according to Vidaver (1967), supplementing the nutrient agar of Difco (Becton-Dickinson, USA) with 5.0 g l^{-1} of mannitol. The plates were incubated at 24 °C for 6 days. Bacterial suspensions in YMA modified medium containing 10^9 cfu ml^{-1} were used to fill the microtubes in aseptic conditions. Strips were incubated at 24 °C for 5, 8 and 12 days. The results of carbon source assimilation were considered positive when the colour turned from green to yellow after 12 days incubation, negative when there was no colour change and weak when the colour turned to pale green but not to yellow. The assimilation of organic salts was considered positive when the medium turned blue, weak when it turned a pale blue and negative if the colour was green.

Table 1. Origin and source of the strains

Strain	Host plant	Location	Source
Clavibacter michiganensis subsp. *sepedonicus*			
94-C3, 95-C19, 95-457, 96-81, 96-100	*Solanum tuberosum*	Spain/Palencia	This study
95-336	*Solanum tuberosum*	Spain/Valladolid	This study
95-C6	*Solanum tuberosum*	Spain/Zamora	This study
95-S18, 95-S29, 95-AS18, 95-AS47, 95-AS48, 95-AS51, 95-AS52, 95-AS78, 95-AS79, 95-AS103, 95-AS115, 95-M6, 95-M207, 96-S49, 96-AS5, 96-M255	*Solanum tuberosum*	Spain/Burgos	This study
96-BA5	*Solanum tuberosum*	Spain/Cáceres	This study
97-GAL1, 97-GAL2	*Solanum tuberosum*	Spain/Pontevedra	This study
C-R2, C-R12, C-R13, C-BRR7	*Solanum tuberosum*	Canada	S.H. de Boer
N-87–5, N-89–4, N-92–5	*Solanum tuberosum*	Norway	A. Sletten
D-221, D-282, D-285, D-288, D-298, D-316 D-320, D-326	*Solanum tuberosum*	Denmark	K. Mansfeld
D-294	*Solanum tuberosum*	Germany	K. Mansfeld
S-175, S-189, S-237, S-247, S-318, S-375, S-379, S-403, S-BD132	*Solanum tuberosum*	Sweden	P. Persson
2140	*Solanum tuberosum*	USA	NCPPB
ATCC33113^T	*Solanum tuberosum*	Canada	NCPPB
Clavibacter michiganensis subsp. *nebraskensis*			
CECT5040^T, CECT4209	*Zea mays*	USA	CECT
Clavibacter michiganensis subsp *tessellarius*			
CECT4263	*Triticum aestivum*		CECT
Clavibacter michiganensis subsp. *insidiosus*			
CFBP5041	*Medicago sativa*	United Kingdom	CFBP
CFBP5042^T	*Medicago sativa*	USA	CFBP
Clavibacter michiganensis subsp. *michiganensis*			
121.1, 093.3F	*Lycopersicon esculentum*	Spain	IVIA
Curtobacterium flaccumfaciens pv. *flaccumfaciens*			
1844, 1412	*Phaseolus vulgaris*	USA	NCPPB
Erwinia (Pectobacterium) carotovora subsp. *atroseptica*			
1001	*Solanum tuberosum*	United Kingdom	SCRI
537	*Solanum tuberosum*	Spain	IVIA
Erwinia (Pectobacterium) carotovora subsp. *carotovora*			
194	*Solanum tuberosum*	United Kingdom	SCRI
163	*Solanum tuberosum*	Spain	IVIA
Erwinia (Pectobacterium) crhysanthemi			
482	*Solanum tuberosum*	Netherlands	PD
1500	*Solanum tuberosum*	Spain	IVIA
Ralstonia solanacearum			
NCPPB1493	*Lycopersicon esculentum*	Puerto Rico	NCPPB
96-508	*Solanum tuberosum*	Spain	This study

CECT: Spanish Type Culture Collection (Spain); CFBP: Collection Nationale de Bactéries Phytopathogènes (France); IVIA: Instituto Valenciano de Investigaciones Agrarias (Spain); NCPPB: National Collection of Plant Phytopathogenic Bacteria (UK); PD: Plant-enziektenkundige Dienst (Netherlands); SCRI: Scottish Crop Research Institute (UK).

Assimilation of arbutin and esculin was considered positive when the medium turned brown.

Results obtained using API 50CH are shown in Table 2. Most of the *C. michiganensis* subsp. *sepedonicus* strains (positive result in more than 90% of strains) used the following as a carbon source: L-arabinose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, mannitol, arbutin, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, D-raffinose and D-turanose. Most of the strains (positive result in under 10% of strains) were unable to use the following: glycerol, erythritol, D-arabinose, ribose, L-xylose, adonitol, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, α methyl-D-mannoside, α methyl-D-glucoside, *N*-acetyl glucosamine, melibiose, inulin, melezitose, starch, glycogen, xylitol, β-gentiobiose L-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto gluconate and 5-keto gluconate. Variable results were obtained for β methyl-D-xyloside and amygdalin because less than 80% of strains used them. These results indicate a

Table 2. Carbon source assimilation using API 50CH system

Carbohydrate	% strains of *Cms* with result			*Cms* [1] Profile [a]	Other reference strains profiles[a]								
	–	+	w		*Cmn* [2]	*Cmt* [3]	*Cmi* [4]	*Cmm* [5]	*Cff* [6]	*Pca* [7]	*Pcc* [8]	*Pch* [9]	*Rso*[10]
Control	100.0			–	–	–	–	–	–	–	–	–	–
Glycerol	90.5	9.5		–	+	+	+	+	+	+	+	+	+
Erythritol	100.0			–	–	–	–	–	+	–	–	–	–
D-arabinose	100.0			–	–	–	–	–	–	–	–	+	–
L-arabinose	1.9	94.3	3.8	+	+	+	+	+	+	+	+	+	+
Ribose	94.3	1.9	3.8	–	–	–	–	–	–	+	+	+	–
D-xylose		98.1	1.9	+	+	+	+	+	+	+	+	+	+
L-xylose	98.1		1.9	–	–	–	–	–	–	–	–	–	–
Adonitol	98.1		1.9	–	–	–	–	–	+	–	–	–	–
β-methyl-D-xyloside	3.8	60.4	35.8	V	+	+	+	+	V	–	–	–	–
Galactose		100.0		+	+	+	+	+	+	+	+	+	+
D-glucose		100.0		+	+	+	+	+	+	+	+	+	+
D-fructose		100.0		+	+	+	+	+	+	+	+	+	+
D-manose		100.0		+	+	w	+	+	+	+	+	+	+
L-sorbose	100.0			–	–	–	–	–	–	–	–	–	–
Rhamnose	100.0			–	–	–	–	–	+	+	+	+	–
Dulcitol	100.0			–	–	–	–	–	–	–	–	–	–
Inositol	100.0			–	+	w	V	+	+	+	+	+	+
Manitol		100.0		+	+	+	+	+	+	+	V	+	–
Sorbitol	100.0			–	–	–	–	–	V	–	–	–	–
α-methyl-D-manoside	100.0			–	–	–	w	–	+	–	–	–	–
α-methyl- D-glucoside	100.0			–	–	–	–	–	+	+	–	–	–
N-acetyl glucosamine	100.0			–	–	–	–	–	+	+	V	–	–
Amygdalin	11.3	13.2	75.5	V	+	+	+	+	+	+	+	–	–
Arbutin		100.0		+	+	+	+	+	+	+	+	+	–
Esculin		100.0		+	+	+	+	+	+	+	+	+	–
Salicin		100.0		+	+	+	+	+	+	+	+	V	–
Celobiose		100.0		+	+	+	+	+	+	+	+	+	+
Maltose		100.0		+	+	+	+	+	+	+	–	–	+
Lactose		92.5	7.5	+	+	+	+	+	+	+	+	+	+
Melibiose	98.1	1.9		–	+	–	–	V	+	+	+	+	–
Sucrose		100.0		+	+	+	+	+	+	+	+	+	–
Trehalose	1.9	94.3	3.8	+	+	+	+	V	+	+	+	–	–
Inulin	100.0			–	–	–	–	–	–	–	–	V	–
Melezitose	100.0			–	–	–	–	–	+	–	–	–	–
D-raffinose	1.9	92.4	5.7	+	w	w	–	+	+	+	+	+	–
Starch	100.0			–	–	–	–	–	–	–	–	–	–
Glycogen	100.0			–	–	–	–	–	–	–	–	–	–
Xylitol	98.1	1.9		–	–	–	–	–	V	–	–	–	–
β-gentiobiose	84.9		15.1	[–]	+	w	+	+	–	V	–	–	–
D-turanose		100.0		+	+	+	+	+	+	w	–	–	–
D-lyxose	100.0			–	–	–	–	–	–	–	–	–	–
D-tagatose	100.0			–	–	–	–	–	–	–	–	–	–
D-fucose	100.0			–	–	–	–	–	–	–	–	–	+
L-fucose	98.1	1.9		–	–	–	–	–	–	–	–	–	–
D-arabitol	100.0			–	–	–	–	–	V	–	–	–	–

Table 2. continued

Carbohydrate	% strains of *Cms* with result			*Cms* [1] Profile [a]	Other reference strains profiles[a]								
	−	+	w		*Cmn* [2]	*Cmt* [3]	*Cmi* [4]	*Cmm* [5]	*Cff* [6]	*Pca* [7]	*Pcc* [8]	*Pch* [9]	*Rso*[10]
L-arabitol	100.0			–	–	–	–	–	V	–	–	–	–
Gluconate	100.0			–	–	–	–	–	–	+	–	–	–
2-keto−gluconate	100.0			–	–	–	–	–	–	–	–	–	–
5-keto−gluconate	100.0			–	–	–	–	–	–	–	–	–	–

[a] +, >90% positive results; V, 20–80% positive results; −, <10% positive results; [−], 10–20% positive results.
1. *Cms: Clavibacter michiganensis* subsp. *sepedonicus,* 2. *Cmn: C.m.* subsp. *nebraskensis,* 3. *Cmt: C.m. subsp. tessellarius,* 4. *Cmi: C.m. subsp. insidiosus,* 5. *Cmm: C.m.* subsp. *michiganensis,* 6. *Cff: Curtobacterium flaccumfaciens pv. flaccumfaciens* 7. *Pca: Erwinia (Pectobacterium) carotovora* subsp. *atroseptica,* 8. *Pcc: Ec.* subsp. *carotovora,* 9. *Pch: Erwinia (Pectobacterium) chrysanthemi,* 10. *Rso: Ralstonia solanacearum.*
Shadowed results differ with respect to *Clavibacter michiganensis* subsp. *sepedonicus* profile.

high intraspecific homogeneity in the use of such carbon sources, over 90% of the *C. michiganensis* subsp. *sepedonicus* strains giving the same result independent of their geographical origin. This uniformity in the use of carbon sources contrasts with the data obtained by Harris-Baldwin and Gudmestad (1996) who characterized coryneform phytopathogenic bacteria using the Biolog automated microbial identification system, obtaining a low level of correct identification (36–59%) for *C. michiganensis* subsp. *sepedonicus*. Although the other subspecies of *C. michiganensis* are not potato pathogens, we compared the results for the subspecies *sepedonicus* with those of representative strains from the other subspecies (Table 2). Differences concerning each of the *C. michiganensis* subspecies are listed in Table 2. Also, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, which is a species phylogenetically related to *C. michiganensis*, displayed many differences compared to *C. michiganensis* subsp. *sepedonicus*. Although these results are relevant from a taxonomic point of view, it is very improbable that such microorganisms will be isolated from potatoes. In contrast, some other potato pathogens that may be isolated with *C. michiganensis* subsp. *sepedonicus*, include soft rot *Erwinia* (*Pectobacterium*) and/or *Ralstonia* species. As can be seen in Table 2, these species can easily be differentiated from *C. michiganensis* subsp. *sepedonicus*. The data obtained by using API 50CH are in agreement with the EU current official analysis methodology, which includes the assimilation of different carbon sources for identification of isolates from the subspecies *sepedonicus*, with the advantage of the standardization and simplicity afforded by a commercial system.

The results show that API 50CH can be used as a rapid and simple method to characterize *C. michiganensis* subsp. *sepedonicus*. This is in accordance with the results obtained in recent work in which this system was used to identify non-clinical bacterial isolates belonging to different Gram-positive genera such as *Lactobacillus* (Charteris et al., 2001), *Bacillus* (Haque and Russell, 2005), or *Paenibacillus* (von der Weid et al., 2000). To our knowledge API 50CH system have not previously been described for characterizing *C. michiganensis* subsp. *sepedonicus*. Comparing the results obtained using API 50CH with those obtained by conventional tube assays for assimilation of carbon sources, we observed that API 50CH results concerning the utilization of L-arabinose, ribose, D-fructose, rhamnose, mannitol, melibiose, sucrose and inulin coincided with the results described by Slack (1987). Differences were observed for trehalose utilization which was positive using API 50CH and negative in the aforementioned work. Moreover, the results of glycerol, rhamnose, esculin and starch tests coincided with those described in the official methodology (Anon., 1993); however different results were obtained for salicin utilization, which was positive using the API 50CH system. This lack of agreement may be due to the different media and culture conditions used for the microtubes compared to conventional tubes and to the variation in incubation time, temperature, pH indicator, oxygenation conditions, etc. Nevertheless, the results obtained in this work would

indicate that the API 50CH system could be included in the official methodology recommended by the European Union.

With respect to enzymatic characterization, this was performed by using the API ZYM system, which is a semi-quantitative micromethod designed to detect enzymatic activity. To inoculate the strips, the strains were inoculated on NBY plates (Vidaver, 1967) and incubated at 24 °C for 6 days. Aliquots of 100 μl from aqueous bacterial suspensions containing 10^{10} cfu ml^{-1} were added to each microtube and the strips were incubated for 4 h at 32 °C in a moist chamber. The results were read following the instructions of the commercial manufacturer. Positive results were considered when the amount of hydrolysed substrate was ≥10 nmol.

The results obtained for enzymatic activity of the *C. michiganensis* subsp. *sepedonicus* strains are listed in Table 3. In general, these strains produced esterase, esterase lipase, leucine arylamidase, β-galactosidase and α-glucosidase (positive results in over 90% of strains). There was no production of lipase, trypsin, chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase (positive results in fewer than 10% of strains). The production of β-glucosidase was positive in 85% of strains and weak in the remaining strains. The result for naphthol-AS-BI-phosphohydrolase was negative in 85% of the strains and weak in the remainder of the strains. Phosphatase alkaline, valine arylamidase, cystine arylamidase and phosphatase acid production was variable (positive results in 20–80% of strains). The enzymatic analysis using the API ZYM system showed homogeneity within *C. michiganensis* subsp. *sepedonicus*, but lower than that obtained using the API 50CH system.

These results are in agreement with those obtained by Vantomme et al. (1987), who obtained

Table 3. Enzymatic activities detected by using API ZYM system

Enzyme	% strains of *Cms* with result			*Cms* [1] Profile[a]	Other reference strains profiles[a]								
	–	+	w		*Cmn* [2]	*Cmt* [3]	*Cmi* [4]	*Cmm* [5]	*Cff* [6]	*Pca* [7]	*Pcc* [8]	*Pch* [9]	*Rso*[10]
Control	100			–	–	–	–	–	–	–	–	–	–
Phosphatase alcaline	64.2	7.5	28.3	V	+	+	–	+	–	+	+	+	+
Esterase (C4)		100		+	+	+	+	+	+	–	–	–	+
Esterase lipase (C 8)		100		+	+	+	+	+	+	–	–	–	+
Lipase (C 14)	90.6		9.4	–	–	–	–	–	–	–	–	–	–
Leucine arylamidase		98.1	1.9	+	+	+	+	+	+	+	+	+	+
Valine arylamidase	75.5		24.5	V	–	–	–	–	–	V	–	–	–
Cystine arylamidase		28.3	71.7	V	–	d	+	d	–	–	–	–	–
Trypsin	100			–	–	–	–	–	–	V	–	–	–
α-chymotrypsin	98.1		1.9	–	–	d	–	–	–	–	–	–	–
Phosphatase acid	7.6	22.6	69.8	V	+	+	+	+	+	+	+	+	+
Naphthol-ASBI-phosphohydrolase	84.9		15.1	[–]	–	–	–	–	–	+	+	+	–
α -galactosidase	100			–	V	+	+	+	–	–	–	–	–
β-galactosidase		92.5	7.5	+	V	+	+	+	+	–	–	+	–
β-glucuronidase	100			–	–	–	–	–	–	–	–	–	–
α -glucosidase		100		+	V	+	+	+	+	+	–	–	–
β-glucosidase		84.9	15.1	[+]	–	+	+	+	+	–	–	V	–
N-acetyl-β-glucosaminidase	98.1		1.9	–	–	–	–	–	+	–	–	–	–
α-mannosidase	98.1		1.9	–	–	–	+	–	V	–	–	–	–
α-fucosidase	100			–	–	–	–	–	–	–	–	–	–

[a] +, > 90% positive results; V, 20–80% positive results; –, < 10% positive results; [+], 80–90% positive results; [–] 10–20% positive results.

1. *Cms: Clavibacter michiganensis* subsp. *sepedonicus,* 2. *Cmn: C.m.* subsp. *nebraskensis,* 3. *Cmt: C.m.* subsp. *tessellarius,* 4. *Cmi: C.m.* subsp. *insidiosus,* 5. *Cmm: C.m.* subsp. *michiganensis,* 6. *Cff: Curtobacterium flaccumfaciens pv. flaccumfaciens* 7. *Pca: Erwinia (Pectobacterium) carotovora* subsp. *atroseptica,* 8. *Pcc: Ec.* subsp. *carotovora,* 9. *Pch: Erwinia (Pectobacterium) chrysanthemi,* 10. *Rso: Ralstonia solanacearum.*

Shadowed results differ respect *Clavibacter michiganensis* subsp. *sepedonicus* profile.

a very similar profile with the API ZYM system applied to 79 *C. michiganensis* subsp. *sepedonicus* strains. The homogeneity within this subspecies was also found by De Bruyne et al. (1992) with API ZYM and other experimental enzymatic galleries (aminopeptidases, esterases, oxidases) which enabled *C. michiganensis* subsp. *sepedonicus* to be grouped in one phenon with an internal similarity of 82%. However they did not indicate the results of the enzymatic activity of each species.

The profiles obtained using the API ZYM system for the strains of the other subspecies from *C. michiganensis* and other phytopathogenic species included in this study are shown in Table 3. Each one of these species and subspecies differed from *C. michiganensis* subsp. *sepedonicus* in at least one enzymatic activity. Therefore, the API ZYM system could be useful to differentiate this species from other phylogenetically related bacteria and from other potato pathogens.

For the statistical analysis of carbon source utilization (API 50CH) and enzymatic activities (API ZYM), the results were codified in binary form. A similarity matrix was constructed using the Jaccard's coefficient. UPGMA cluster analysis of the data (Sokal and Michener, 1958) enabled a dendrogram to be constructed (Figure 1). Strains were separated into five groups. Three of them included strains that were different from *C. michiganensis* species. The first one comprised the *Ralstonia solanacearum* strains with similarity levels below 50% as compared to the remaining strains. The second group included the strains of *Erwinia* (*Pectobacterium*) *carotovora* subsp. *atroseptica*, *Erwinia* (*Pectobacterium*) *carotovora* subsp. *carotovora* and *Erwinia* (*Pectobacterium*)

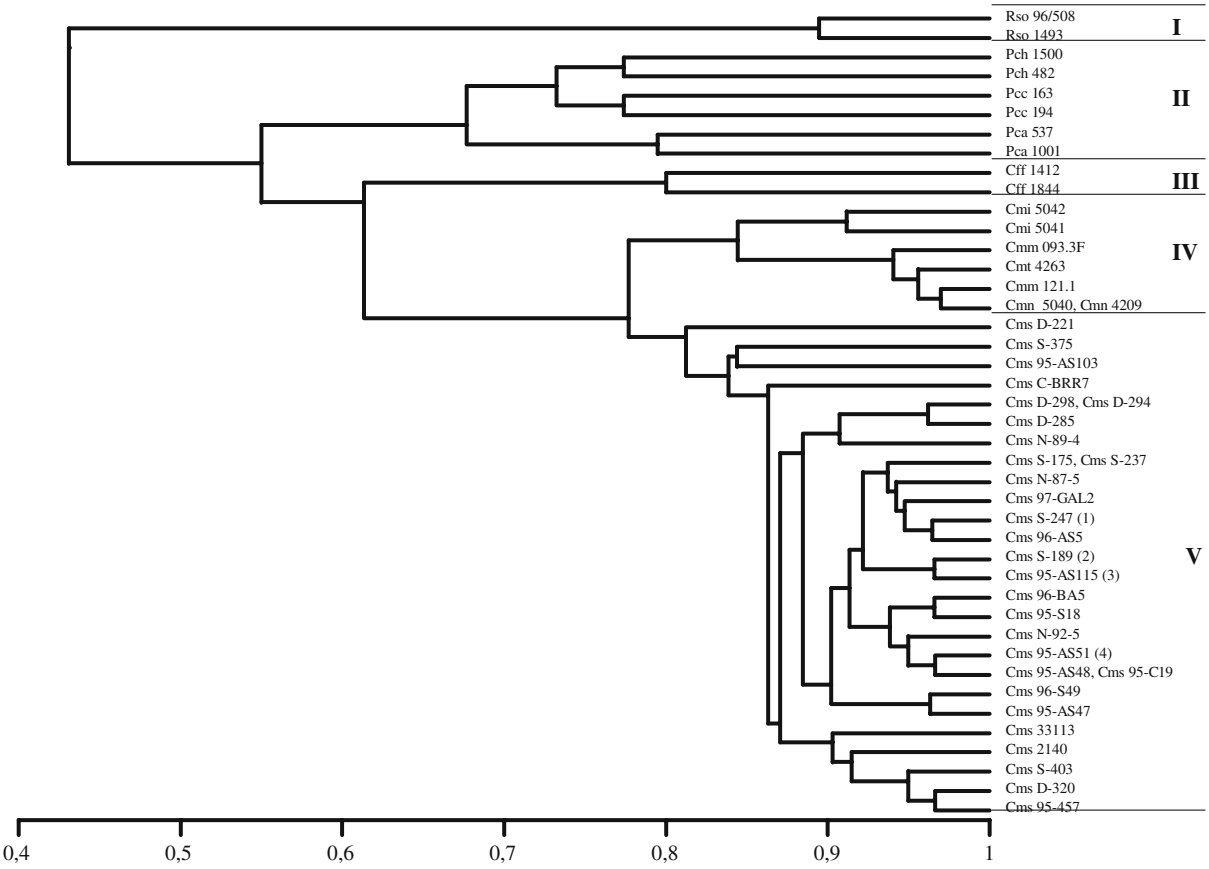

Figure 1. Dendrogram based on API 50CH and API ZYM results. Species abbreviations are similar to Tables 1 and 2. Group V(1) includes strains: S-BD132, D-316, D-288, D-282, CR-2, CR-13, CR-12, 96-M255, 96–100, 95-AS79, 95-AS78, 95-AS18, 95–336, 95-M6; Group V(2) includes strains: D-326, 95-S29, 95-AS52, 95-M207; Group V(3) includes strains: S-379, S-318, 97-GaL1, 96–81; Group V(4) includes strains: 95-C6, 94-C3.

chrysanthemi. The third one was formed by the *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* strains. A fourth group comprised *C. michiganensis* subsp. *nebraskensis*, *michiganensis*, *tessellarius* and *insidiosus*, with a similarity coefficient of over 84%. A fifth group included the 53 strains of *C. michiganensis* subsp. *sepedonicus* with an internal similarity coefficient higher than 80% irrespective of their geographic origin. This last group was clearly different from the previous one, with a similarity coefficient below 80%.

All these data strongly support the use of API 50CH and API ZYM for accurate characterization of *C. michiganensis* subsp. *sepedonicus* and their inclusion in the official methodology for its identification. The results obtained with the two commercial systems analysed support the current classification of the species *C. michiganensis*. They also showed that there was a clear separation between the subspecies *sepedonicus* and the remaining subspecies belonging to the same species. These results are in agreement with those obtained using 16S rRNA sequencing, DNA–DNA hybridization (Döpfer et al., 1982), LMW RNA analysis (Palomo et al., 2000), TP-RAPD profiling (Rivas et al., 2002), Rep-PCR (Smith et al., 2001), serological analysis (Lazar, 1968) and enzymatic studies (De Bruyne et al., 1992).

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