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Short communication

Identification and characterisation of bacteria causing soft-rot in *Agave tequilana*

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Abstract

Agave tequilana is the raw material for the production of the alcoholic beverage tequila. A bacterial disease has affected the *A. tequilana* crop in recent years. Previous reports based on colony and cell morphology, Gram stain and potato rot indicated that *Erwinia* sp. is the main pathogen. We isolated a several bacterial isolates capable of producing soft-rot symptoms in greenhouse pathogenicity assays. An extensive characterisation involving pathogenicity tests, fatty acid profile, metabolic and physiological properties, ribosomal DNA sequence and intergenic transcribed spacer amplification (ITS-PCR) and restriction banding pattern (ITS-RFLP) was made of each isolate. Three different species: *Erwinia cacticida*, *Pantoea agglomerans* and *Pseudomonas* sp. were identified. Fatty acid and metabolic profiles gave low similarity values of identification but 16S rDNA sequence, ITS-PCR and ITS-RFLP confirmed the identification of *E. cacticida*. In the phylogenetic tree, *E. cacticida* from blue agave was grouped neither with *E. cacticida* type strains nor with *Erwinia carotovora*. This is the first report that associates *E. cacticida* with *A. tequilana* soft-rot symptoms.

Introduction

Agave tequilana Weber (blue agave) is the raw material for producing the alcoholic beverage tequila. According to international law agreements, tequila can only be produced in a limited region of Mexico, including the states of Guanajuato, Jalisco, Michoacan, Nayarit and Tamaulipas (SCFI, 1997). In 1998, total agave production comprised 203 million plants, with the State of Jalisco as the main producer (Valenzuela-Zapata, 1994; Ramírez, 1998). Tequila production has risen since 1991 from 86 million litres to 156.4 million litres in 1998 (Rivera, 1998; Frias, 1999). Similarly, employment in the tequila industry has risen since 1984 from 17,130 employees to about 38,000 employees at present (Rivera, 1998; Frias, 1999; Miller, 2000).

A bacterial disease causing serious losses in commercial *A. tequilana* production was first reported in 1989. The soft-rot symptoms of this disease were similar to those produced by *Erwinia* sp. (Valenzuela-Zapata, 1994). Other micro-organisms have also been isolated from roots and stem (*Verticillium* sp. and *Fusarium* sp.) and leaves (*Asterina mexicana*, *Fusarium* sp.). (Larrea-Reynoso, 1998; Martínez-Ramírez and Virgen-Calleros, 1998). Most studies have singled out *Erwinia carotovora* as the sole soft-rot pathogen of *A. tequilana*. This disease has led to a recent shortage in agave, and as a consequence tequila production fell from 200 million litres in 1999 to 181.6 million litres in 2000. The shortage has also driven ∼80 tequila manufacturers to suspend production of tequila derived entirely from *A. tequilana*

(Rivera, 1998; Frias, 1999). The reduction in the production of agave is due mainly to two diseases that can be present simultaneously: one affects roots and is believed to be caused by *Fusarium oxysporum*, while the other affects leaves and is proposed to be caused by soft-rot bacteria. These diseases are present in ∼30% of the plants (Alegria and González, 1998). The lost revenue due to the disease reached $200 million in 1999 (Alegria and González, 1998; Frias, 1999).

The taxonomy of the genus *Erwinia* has been complicated by the heterogeneity of the strains included in the taxon (Holt et al., 1994) and has been re-classified in different groups based on biochemical and DNA characteristics (Kwon et al., 1997; Hauben et al., 1998; Spröer et al., 1999). It has been proposed that *Erwinia* should be limited to pathogens that cause wilt diseases (*Erwinia amylovora* and *Erwinia tracheiphila*), with the more active soft-rot Erwinias placed in the separate genus *Pectobacterium* (Beji et al., 1988; Hauben et al., 1998). Both *Erwinia* and *Pectobacterium* are valid names for this group of pathogens but in this paper we will use *Erwinia*. The new genus *Pantoea* includes strains of the former species *Erwinia herbicola* and *Enterobacter agglomerans*. Despite these changes, there are species of *Erwinia* that have characteristics that are not strictly from one group or the other (Beji et al., 1988; Hauben et al., 1998; Holt et al., 1994; Kwon et al., 1997; Spröer et al., 1999).

In this work, *Erwinia cacticida* was identified as one of the pathogens associated with soft-rot symptoms. We also identified *Pantoea agglomerans* (synonym *Erwinia agglomerans*) and *Pseudomonas* sp. present in leaves showing soft-rot symptoms.

Materials and methods

Isolation of bacteria from plants in the field. Bacteria were isolated from *A. tequilana* plants collected in Tequila, State of Jalisco, Mexico. Plant age ranged from less than one year to seven years. Plant samples were obtained from random sites in the field. A total of 72 leaves were sampled on three sampling dates, two in the rainy season and another in the dry season. Small portions of leave tissue, ca. 1.0 cm^2 (3 portions/leaf) were disinfected using sodium hypochloride 2% (v/v) and ethanol 70% (v/v), and washed twice with sterile distilled water. These tissue portions were transferred to nutrient agar plates and incubated at 28–30 °C for 24–48 h. Bacterial colonies were picked at random from the plates, checked for purity and grouped according to colony colour and morphology, cell shape, growth rate and Gram reaction. Based on these parameters, 15 different bacteria were identified. These bacteria were the most frequently isolated.

Reference bacterial isolates of *Erwinia carotovora* subsp. *carotovora* 71, *Pseudomonas syringae* pv. *phaseolicola* and *E. agglomerans* (synonym *P. agglomerans*) donated by the University of Missouri, USA; Cinvestav–Irapuato and CIDIIR, Mexico, respectively, were used in establishing the identity of the bacterial isolates.

Culture and maintenance of isolates. Bacteria were cultured on nutrient agar and only Gram-negative isolates were subsequently studied considering that soft-rot symptoms of *A. tequilana* were associated to *E. carotovora*. The bacterial isolates were cultured in Luria–Bertani (LB) broth and for maintenance in nutrient broth–glycerol (8 : 2 (v/v) and frozen at −70 °C) and French agar (nutrient broth, 8 g l^{-1}; thiamine, 80 mg l^{-1} and agar, 7.6 g l^{-1}).

Preparation of inoculum and inoculation of agave plants. An aqueous bacterial suspension of 1.5×10^8 CFU ml^{-1} was prepared in sterile distilled water. Aliquots of 100 µl of the suspension were injected subcuticularly into the youngest leaves (Isakeit et al., 1997). Prior to inoculation, leaf surfaces were disinfected with ethanol 70% (v/v). Inoculated plants were covered with polypropylene bags during the first 30 days to increase the relative humidity of the environment surrounding the plants. Plants used as negative controls were inoculated with 100 µl of sterile distilled water. An additional set of negative controls consisted of plants inoculated with *E. agglomerans* and *P. syringae* pv. *phaseolicola*. Disease development was monitored for 70 days.

Pathogenicity test in diverse hosts. Isolates causing soft-rot symptoms were tested for pathogenicity in hosts other than blue agave.

Pepper (cv. California Wonder), tomato (cv. Rio Fuego) and cucumber (Poinset) fruits, potato tubers (cv. Alpha) and onion bulbs (cv. Texas Early White), were immersed in 2% aqueous solution of sodium hypochloride for 2 min, then in ethanol 70% for 2 min, rinsed in deionized sterile water and air dried.

Fruits of bell pepper and tomato were inoculated by punctures made with four straight 2 mm pins, and

inoculated with 50 µl of an aqueous cell suspension of bacteria (10^8 CFU ml^{-1}) (Lapwood et al., 1984; Smith and Bartz, 1990; Stommel et al., 1996). Five punctures per fruit and four replicates for each strain were included. Punctures were covered with filter paper (7 mm in diameter) after inoculation. They were incubated at 30 °C and >85% relative humidity (RH) for about 72 h. Potato tubers, onion bulbs and cucumber fruits were wounded (without removing the tissue debris) with a flamed cork borer (10 mm in diameter and 5 mm in depth) and inoculated with 50 µl of aqueous bacterial suspension (10^8 CFU ml^{-1}) (Lapwood et al., 1984; Smith and Bartz, 1990). Five wounds per fruit were made and four fruit replicates for each strain. Potato tubers were disinfected before cutting off 10 mm thick slices. Wounds, 5 mm in depth and 10 mm in diameter were made by pressing a cork borer onto the surface of each slice. Three wounds per slice and four slice replicates for each strain were tested. Symptoms were monitored for 72 h.

Two species of cactus (*Ferocactus latispinus* and *Mammillaria san-angelensis*) and a second agave species (*Agave atrovirens*) were also inoculated (Isakeit et al., 1997). Leaf surfaces were disinfected and injected subcuticularly into the cacti and the youngest agave leaves with bacterial suspension. Five wounds per plant were made and three plant replicates for each strain. Disease development was monitored for 30 days.

Morphological description. Bacterial cultures (18-h-old) of agave were streaked on LB agar medium and incubated for 24 h at 28 °C for colony characterisation. They were also cultured on crystal violet pectate (CVP) and potato-dextrose peptone (PDP), and were incubated for 24 h at 28 and 43 °C, respectively, for differentiating *E. carotovora* from *E. cacticida* strains (Dhingra and Sinclair, 1986; Alcorn et al., 1991). In order to determine cell morphology, bacterial isolates were cultured on LB at 28 °C for 24 h.

Electron microscopy. Bacterial cultures (24-h-old) were washed in distilled water. A 2 : 3 dilution (bacterial suspension: water) was prepared for each isolate. One drop of the dilution was placed on a microscope grid; it was air-dried and coated with gold in an E.M. Fullan EMS-76M evaporator for 3 min. Mounts were examined in a Philips XL 30 ESEM scanning electron microscope operated at 4462× magnification and at 20.0 kV.

Antibiotic resistance. To screen for antibiotic resistance to differentiate between closely related micro-organisms, isolates were spread over potato–dextrose agar (PDA) medium for 18–20 h at room temperature as described previously (Maniatis et al., 1989). The antibiotics tested were penicillin, enoxacin, netylmycin, ceftriaxone, trimethoprim sulphamethoxyacid, erythromycin, cephalotin, chloramphenicol, amykacin and gentamycin. (Sanofi Diagnostics Pasteur Inc., Redmond, WA).

Fatty acids profile. The fatty acids profiles were performed at the Central Science Laboratory (CSL), York, UK. The identification of fatty acids profile was done with the bacterial isolates that produced disease symptoms on blue agave in greenhouse experiments. It was based on TSBA40 and NCPPB3 libraries. Bacterial isolates were grown in BBL trypticase soy broth agar (Becton Dickinson Co. Cockeysville, MD) at 28 °C for 24 h. Bacterial cells were saponified in NaOH and methanol. The fatty acids were esterified with HCl and methanol, extracted in solvent (methyl-ter-butyl-ether and hexane), washed in a weak NaOH solution and separated in an HP 6890 gas chromatograph (DeBoer and Sasser, 1986) and identified with MIDI database (Newark, Delaware, USA).

Metabolic and physiological characteristics. The biochemical properties of the isolates were determined by their ability to oxidise carbon sources using the Microlog GN2 microplates system (Lee et al., 1997; Wilson et al., 1999; Duncan et al., 2002; Gilho et al., 2002). The bacterial isolates were grown on Biolog universal growth medium (BUGM) (Biolog Inc., Hayward, CA) and incubated at 28 °C for 18 h. They were also tested for catalase, oxidase, nitrate reduction, methyl red, Voges–Proskauer, ornithine decarboxylase, hydrogen sulphide on TSI, motility and gas production from D-glucose.

Pectolitic activity was determined by growing the bacteria in pectate medium (Hildebrand, 1971) at two different pH levels (5.0 and 7.0). On CVP, two different temperatures were tested (28 and 43 °C).

Ribosomal sequences (16S rDNA). DNA was extracted from the pathogenic isolates (Maniatis et al., 1989). Polymerase chain reaction (PCR) amplification of 16S rDNA was made using universal primers (Widmer et al., 1998). The fragment amplified was about 900 bp in length. It was subsequently cloned into

the pCR 2.1 TOPO vector (Invitrogen Corporation Carlsbad, CA, USA) for sequencing. DNA sequencing was done in the sequencing laboratory at Cinvestav–Irapuato, Mexico.

Sequence data analysis. Alignments of 40 16S rDNA sequences were performed using the ClustalX algorithm (Thompson et al., 1997) with a Gap opening of 10, Gap Extension of 0.2 and DNA transition weight of 0.5. A tree was constructed using the neighbour-joining (Saitou and Nei, 1987), and the Felsenstein confidence limits of it, was assessed by 1000 bootstrap replications (Felsenstein, 1985). Sequences of the type strains used in this study were taken from the GenBank database.

Intergenic transcribed spacer (16S–23S rRNA). The multicopy intergenic transcribed spacer (ITS) was amplified using the primers G1 and L1 (Toth et al., 2001). The amplification conditions were as follows: 95 for 10 min, 30 amplification cycles were performed at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and a final extension of 7 min at 72 °C. Three micro litre of the amplified product was digested with *Rsa*I (Invitrogen Co., Carlsbad, CA, USA) as described by the manufacturer. Banding patterns were observed on 2% NuSieve GTG agarose gel (Flowgen, Ashiby de la Zouch, UK) in TBE.

Results

Sample collection. The study was done during 2000–2001. Three sampling dates were done, two in raining season (summer of 2000 and 2001) because during raining season the incidence of the disease increases, and another in dry season (fall of 2001). A total of 72 leaves were collected per sampling date. About 80% of the leaves showed soft-rot symptoms and from these, different bacteria were isolated. The average number of bacteria isolated from each leaf was 10. Leaves collected from raining season had colonies matching one of the four soft-rot species identified, while leaves of the dry season matched for three of the four soft-rot species.

Greenhouse pathogenicity tests. Soft-rot symptoms observed in blue agave in the field (Figure 1A,B) were reproduced in greenhouse experiments (Figure 1C–F). Fifteen different bacterial isolates were collected from blue agave, but not all were capable of causing disease symptoms in greenhouse experiments. Only four isolates caused disease and were re-isolated from leaves showing soft-rot symptoms for completing Koch's postulates.

Monitoring of blue agave plants revealed chlorotic lesions around the inoculation site. As the disease progressed, between 15 and 20 days after inoculation, different types of symptoms were observed: dark green lesions, chlorotic and brown spots, and soft-rot symptoms (Figure 1F). At 30 days after inoculation, some chlorotic lesions became light brown and were contained within a chlorotic margin, while others turned soft and browner (Figure 1C). At 70 days after inoculation, lesions were drier and became dark brown (Figure 1D,E). No symptoms were observed on control plants, nor on control agave plants inoculated with reference bacteria isolates (data not shown) *E. carotovora* subsp. *carotovora* 71, *E. agglomerans* and *P. syringae* pv. *phaseolicola* or with sterile distilled water (Figure 1G). Isolate IX (Figure 1F) produced the most severe soft-rot symptoms in a short time (within 15 days) compared to isolates I (Figure 1C) and III (Figure 1D). Isolated VII (Figure 1E) was the slowest and less severe. Two different tests were done in two separate experiments, five plants per isolate were used in each one with five replicates per leaf and five leaves per plant.

Pathogenicity on diverse hosts. Bacterial isolates of *A. tequilana* found to cause soft rot in its leaves were tested for pathogenicity in bell pepper, tomato, cucumber, potato and onion. Isolate IX was capable of causing soft rot in fruits of pepper, tomato and cucumber, and in potato tubers and potato slices 72 h after inoculation (Figure 2A–D). The symptoms were more severe than those caused by *E. carotovora* subsp. *carotovora* (Figure 2A–D). Soft-rot symptoms were observed at the same time with both bacteria but tissue maceration was clearly evident with isolated IX. It also caused soft rot on *M. san-angelensis* 15 days after inoculation (Figure 2E). Isolates I, III, VII and *E. agglomerans* were not pathogenic to any of these hosts.

Isolate description. Bacterial isolates pathogenic on blue agave revealed considerable genetic and biochemical differences. These are described below.

Isolate I and isolate III. Ribosomal DNA sequences (fragment of 900 bp) demonstrated that bacterial isolates I and III correspond to *P. agglomerans*. They showed differences in three base positions: 548, 550

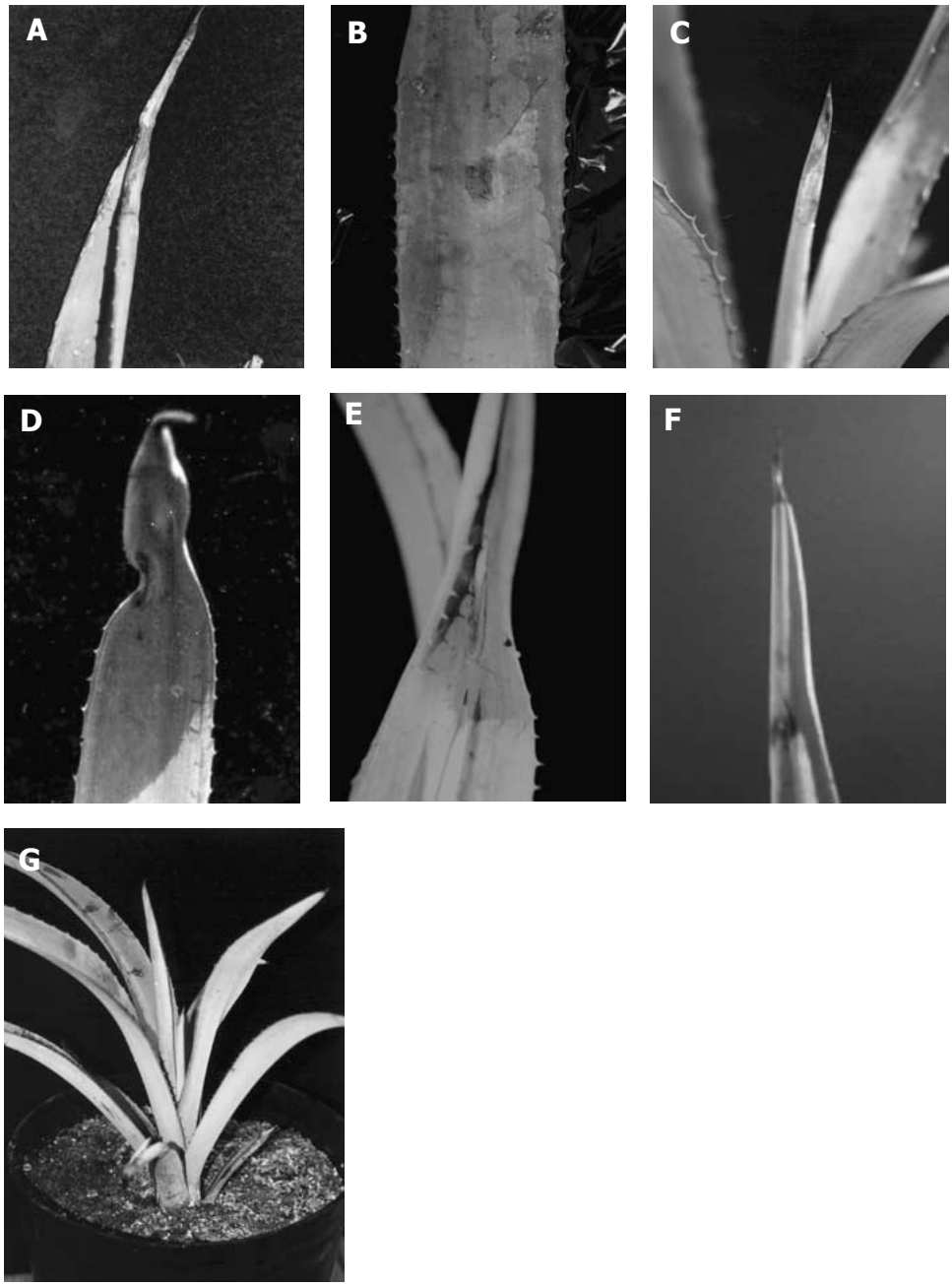

Figure 1. Disease symptoms associated with blue agave bacterial isolates. A–B: soft-rot symptoms observed in field. C–F: symptoms on leaves in greenhouse experiments. C–E: soft-rot symptoms caused by isolates I and III, and VII, respectively. F: Symptoms caused by isolate IX 15 days after inoculation. G: leaves inoculated with sterile ddH_2O.

and 853. The bases are C, T, A and T, A, R in isolate I and isolate III, respectively. The partial ribosomal DNA sequence of both isolates, were submitted to GenBank, (accession numbers are AF498639 and AY88305, respectively). They had 99% homology with *P. agglomerans*, and 98% homology with *Enterobacter cloacae*, *E. dissolvens*, *Salmonella* spp. and *Citrobacter* spp.

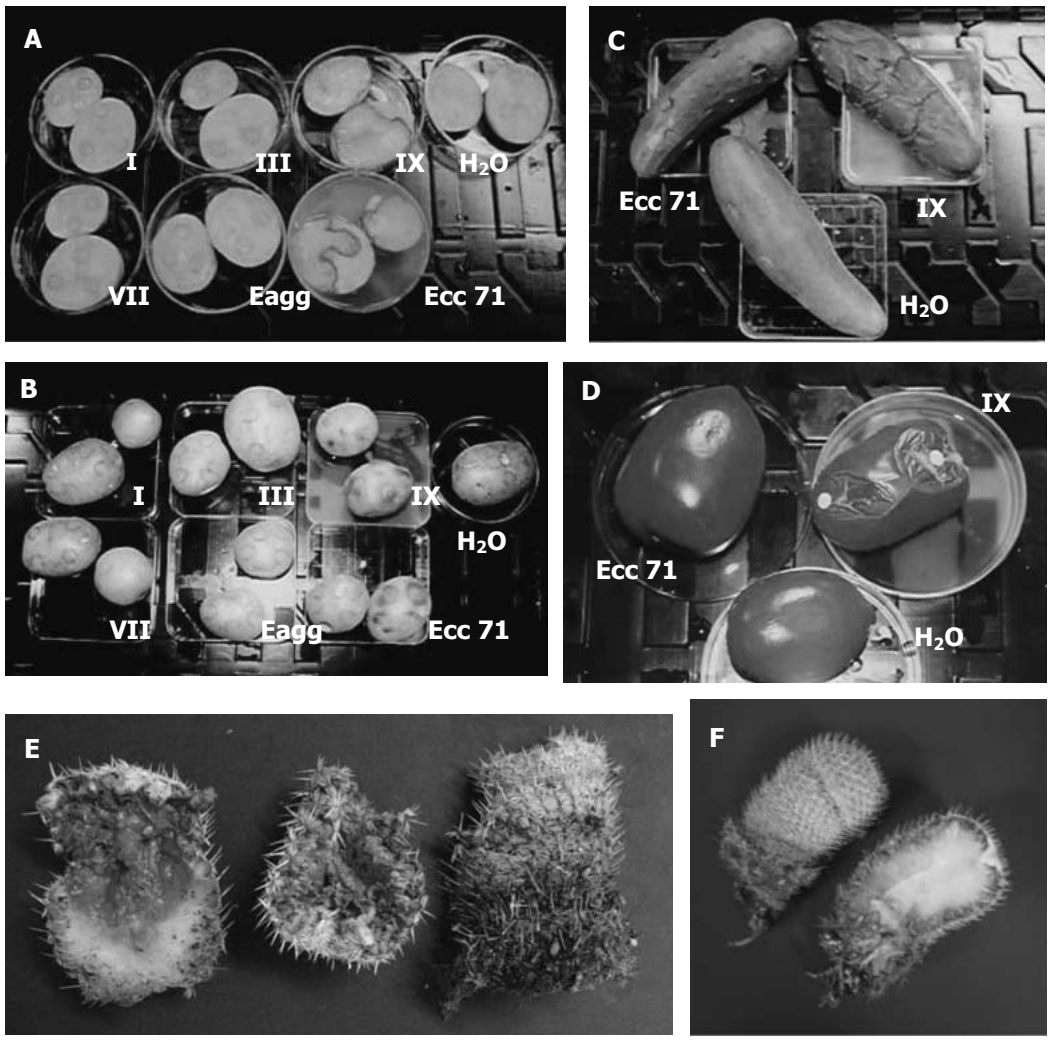

Figure 2. Soft-rot symptoms caused by blue agave bacterial isolates in different hosts. A–D: tissue maceration in potato, tomato and cucumber caused by isolate IX and Ecc71 (*E. carotovora* subsp. *carotovora*). E: symptoms in *M. san-angelensis* 15 days after inoculation caused by isolate IX.

The fatty acid profile together with the biochemical and physiological properties confirmed that isolates I and III correspond to *Pantoea agglomerans*. The fatty acids profiles were 90.2% (isolate I) and 78.7% (isolate III) similar to *P. agglomerans*.

Through the paper will refer to them as *P. agglomerans* I and *P. agglomerans* III. They oxidised D-fructose, D-galactose, L-arabinose, D-mannitol, D-mannose, D-psicose, sucrose, D-trehalose, L-serine, glycerol, N-acetyl-D-glucosamine, cellobiose, maltose, L-rhamnose and L-glutamic, but did not oxidised raffinose, melibiose, adonitol or L-fucose (Table 1). Although *P. agglomerans* I and III are the same species, they have different biochemical profiles and can be considered as different strains. *P. agglomerans* I can oxidise D-serine, L-threonine and methyl pyruvate while *P. agglomerans* III can oxidise D, L-alanine, L-histidine, α-D-glucose, α-keto-glutaric acid and L-alanyl glycine (Table 1). Although they shared some of the metabolic characteristics of the type strain *E. agglomerans*, they were different from this bacterium in its inability to oxidise lactose, malonic acid, L-threonine, α-hydroxybutyric acid and ρ-hydroxyphenyl acid (Table 1). Both isolates are

Table 1. Metabolic and physiological characteristics for bacterial isolates pathogenic to leaves of *A. tequilana*[a]

Substrate	Isolates						
	I	III	VII	IX	Ecc71	Eagg	Psp
Dextrin	+	+	−	+	+	+	−
Glycogen	−	−	−	−	−	+	+
Tween-40	−	−	−	−	−	+	−
Tween-80	−	−	−	+	−	−	−
N-acetyl-D-galactosamine	−	−	−	−	−	−	−
N-acetyl-D-glucosamine	+	+	−	+	+	+	−
Adonitol	−	−	−	−	−	−	−
L-arabinose	+	+	−	+	+	+	−
Cellobiose	+	+	−	+	+	+	−
D-fructose	+	+	−	+	+	+	+
L-fucose	−	−	−	−	−	+	−
D-galactose	+	+	−	+	+	+	−
Gentibiose	+	+	−	+	+	+	−
α-D-glucose	−	+	−	+	+	−	+
m-inositol	−	−	−	+	+	−	−
α-D-lactose	−	−	−	+	+	+	−
Lactulose	−	−	−	−	+	−	−
Maltose	+	+	−	−	−	+	−
D-mannitol	+	+	−	+	+	+	−
D-mannose	+	+	−	+	+	+	−
D-melibiose	−	−	−	+	+	+	−
β-methyl-D-glucoside	+	+	−	+	+	+	−
D-psicose	+	+	−	+	+	+	−
D-raffinose	−	−	−	+	+	−	+
L-rhamnose	+	+	−	+	+	+	−
D-sorbitol	+	+	−	−	−	+	−
Sucrose	+	+	−	+	+	+	+
D-trehalose	+	+	−	+	+	−	−
Xylitol	−	−	−	−	−	−	−
Methyl pyruvate	+	−	−	+	+	+	−
Acetic acid	−	−	+	−	−	+	−
Citric acid	+	+	+	−	−	+	−
Formic acid	+	+	−	−	+	−	+
D-galacturonic acid	+	+	−	−	−	+	−
D-gluconic acid	−	+	−	−	−	+	+
D-glucosaminic acid	−	−	+	−	−	−	−
D-glucoronic acid	+	+	−	−	−	+	−
α-hydroxybutyric acid	−	−	−	−	−	+	−
ρ-hydroxyphenyl acid	−	−	−	−	−	+	−
α-keto-glutaric acid	−	+	+	−	−	−	−
D,L-lactic acid	+	+	+	+	−	+	−
Malonic acid	−	−	−	−	−	+	−
D-saccharic acid	+	+	−	+	+	+	+
Succinic acid	+	+	−	+	+	+	+
D-alanine	−	+	−	−	−	+	+
L-alanine	−	+	−	−	−	+	+
L-alanyl glycine	−	+	−	−	−	+	+
L-asparagine	+	+	+	+	+	+	+
L-aspartic acid	+	+	+	+	+	+	+
L-glutamic acid	+	+	−	−	−	+	+
L-histidine	−	+	−	−	−	+	−
Hydroxy-L-proline	−	−	−	−	−	−	−

Table 1. Continued

Substrate	Isolates						
	I	III	VII	IX	Ecc71	Eagg	Psp
L-proline	−	−	−	−	−	−	−
L-phenylalanine	−	−	−	−	−	−	−
D-serine	+	−	−	−	−	−	−
L-serine	+	+	+	+	+	+	+
L-threonine	−	−	−	−	−	+	−
γ-amino butyric acid	−	−	−	−	−	−	+
Inosine	+	+	−	−	+	+	−
Glycerol	+	+	−	+	+	+	+
Glucose 1-phosphate	+	+	−	+	+	+	−
Glucose 6-phosphate	+	+	−	+	+	+	−
D-arabitol	−	−	−	−	−	−	−
L-leucine	−	−	−	−	−	−	−
L-ornithine	−	−	−	−	−	+	−
Phenylethylamine	−	−	−	−	−	−	−
Putrescine	−	−	−	−	−	−	−
Physiological characteristics[b]	I	III	VII	IX	Ecc71	Eagg	Pseud.
Catalase	+	+	+	+	+	+	+
Oxidase	−	−	+	−	−	−	+
Vogues–Proskauer	+	+	+	+	−	+	+
Methyl red	−	−	−	+	−	±[c]	+
H_2S on TSI	−	−	−	−	−	−	−
Urea	−	−	+	−	−	−	
Ornithine Decarboxylase	−	−	−	−	−	±[c]	−
Motility	+	+	−	−	+	+	+
D-Glu, gas production	+	+	−	+	+	+	
Malonate utilisation	−	−	−	−	±[c]		
Indole	+	+	−	+	−	−	
NO_3	+	+	−	+	−	+	
Pectate	+	+	−	+	+	−	−
Growth at 43 °C	+	+	−	+	+	−	−[d]

[a]Isolates I, III, VII and IX isolated from lesions on leaves of *A. tequilana*; Ecc71 = *E. carotovora* subsp. *carotovora*; Eagg = *E. agglomerans*: Psp = *P. syringae* pv. *phaseolicola*.
[b]Physiological characteristics of type strains taken from Holt et al., 1994; Iimura and Hosono, 1996 and Gavini et al., 1989. Pseud = Pseudomonaceae.
[c]Reaction variable (Holt et al., 1994).
[d]*P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes* can grow at 41 °C.

positive for catalase, Vogues–Proskauer, and malonate and pectate utilisation. They grew on PDP at 43 °C and were oxidase negative (Table 1).

Both isolates were identified as the same species therefore antibiotic resistance profile was evaluated in order to separate them into strains. *P. agglomerans* I and III were very sensitive to ceftriaxone and trimethoprim sulphamethoxyacid, but they differed in their sensitivity to erythromycin and chloramphenicol (Table 2).

Different colony morphologies were also observed on LB, CVP and PDP media. On LB medium, both isolates had circular colonies with a creamy colour. On CVP medium, *P. agglomerans* I and III had circular, smooth and concave colonies while on PDP, they had yellow circular colonies. Cells of both *P. agglomerans* isolates were rod shaped and measured ca. 0.62 µm by 1.4 µm.

Isolate VII. The 16S ribosomal DNA sequence showed that isolate VII is a *Pseudomonas* sp. (GenBank accession number AF498638). It had 97% homology with *Pseudomonas mendocina* and *P. alcalophyla*, and 96% similarity to *P. pseudoalcaligenes*. This result correlated with the identification done by biochemical methods, and confirmed that this bacterium is a *Pseudomonas* sp.

Table 2. Antibiotic resistance profiles[a] of reference bacterial isolates and bacterial isolates determined to cause soft-rot disease on greenhouse inoculated leaves of *A. tequilana*

Isolate[c]	Antibiotic[b]									
	PE	ENX	NET	CRO	SXT	E	CF	CL	AK	GE
I	−	+	−	—	—	—	−	−	+	+
III	−	−	−	—	—	−	−	—	−	−
VII	+	+	+	+	+	+	+	+	+	+
IX	+	+	+	−	+	+	+	+	+	+
Ecc71	—	—	—	—	—	—	—	—	—	—
Eagg	+	+	+	+	+	+	+	+	+	+
Psp	−	−	+	+	+	+	+	−	−	+

[a]+ = resistant (No zone of inhibition); − = sensitive (diameter of zone of inhibition = 1.5–2.4 cm); — = diameter of zone of inhibition = >2.4 cm.
[b]PE = penicillin (10 U); ENX = enoxacin (10 μg); NET = netylmycin (30 μg); CRO = ceftriaxone (30 μg); SXT = trimethoprim sulphamethoxyacid (25 μg); E = erythromycin (15 μg); CF = cephalotin (30 μg); CL = chloramphenicol (30 μg); AK = amykacin (30 μg); GE = gentamycin (10 μg).
[c]Ecc71 = *E. carotovora* subsp. *carotovora*; Eagg = *E. agglomerans*; Psp = *P. syringae* pv. *phaseolicola*.

Pseudomonas sp. isolated from *A. tequilana* was identified by fatty acids profile analysis as *P. mendocina* (60.4% similarity) characterised by small quantity of 10 : 2OH. It also had 47.8% and 44.6% similarity to *P. alcaligenes* and *P. pseudoalcaligenes*, respectively. It had a profile typical of *Pseudomonas* sp., and was considerably different from profiles observed for *P. agglomerans* I and III and isolate IX (Table 1). It oxidised acetic acid, cis-acotinic acid, citric acid, D-glucosaminic acid, α-ketoglutaric acid, D,L-lactic acid, L-serine, L-asparagine and L-aspartic acid, as did the reference isolates *P. syringae* pv. *phaseolicola* (Table 1) and *P. mendocina*, but unlike *P. alcaligenes* and *P. pseudoalcaligenes* (Holt et al., 1994). It was Vogues–Proskauer and utilised urea, but did not use malonate or reduce nitrate (Table 1).

The *Pseudomonas* sp. isolated from blue agave differed from the reference isolate *P. syringae* pv. *phaseolicola* in its antibiotic resistance profile (Table 2); being sensitive to penicillin, enoxacin, chloramphenicol and amykacin.

Colony morphology varied depending on the medium used. *Pseudomonas* sp. produced yellow colonies with an irregular shape on LB medium, where as on PDP medium it produced yellow circular colonies. *Pseudomonas* spp. can be differentiated on their ability to produce pigment on King's B medium (KB) (Holt et al., 1994), so *Pseudomonas* sp. was grown on KB at 28 °C for 24 h and it produced yellow fluorescent pigment. A yellow cellular pigment is also produced by *P. mendocina* (although it is not fluorescent), but not by *P. alcaligenes* and *P. pseudoalcaligenes* (Holt et al., 1994); therefore it might be a type of fluorescent *Pseudomonas*. On CVP medium, *Pseudomonas* sp. did not grow. Its cells are straight rods measuring ca. 0.43 μm by 1.7 μm.

Table 3. Fatty acid ratios of *Erwinia* sp. and bacterial isolates found to cause soft-rot disease on leaves of *A. tequilana*

Isolate[a]	12 : 0/14 : 0	16 : 0/12 : 0	16 : 1/18 : 1
I	0.57	8.83	0.95
II	ND[c]	9.02	1.15
VII	26.89	2.37	0.45
IX	3.83	4.68	1.05
Ecc[b]	4.09–8.86	3.0–4.78	1.19–2.13

[a]Isolates I, III, VII and IX isolated from lesions on leaves of *A. tequilana*; Ecc – *Erwinia carotovora* subsp. *carotovora*.
[b]Reported by DeBoer and Sasser (1986).
[c]Not determined.

Isolate IX. Based on the sequence of a fragment of ribosomal DNA (GenBank accession number AF498637), isolate IX was identified as *E. cacticida* (98% homology). We also compared it with another ribosomal DNA fragment (615 bp) (GenBank accession number AY293739) and it had 100% homology to *E. cacticida* and 99% similarity to *E. carotovora*. The fatty acids analysis correlated with DNA identification despite giving a low match with *E. cacticida* (44.5%). This suggests that isolate IX might be an aberrant strain of *E. cacticida*. Fatty acids ratios: 12 : 0/14 : 0; 16 : 0/12 : 0 and 16 : 1/18 : 1, demonstrated isolate IX is different from *E. carotovora* (Table 3); even though it matched the ratio 16 : 0/12 : 0.

Erwinia cacticida oxidised D-fructose, D-galactose, L-arabinose, D-mannitol, D-mannose, D-psicose, sucrose, D-trehalose, L-serine, glycerol, cellobiose, raffinose, maltose, malonate, gentibiose, *m*-inositol and *N*-acetyl-D-glucosamine but did not oxidised formate, lactulose and inosine as *E. carotovora* subsp. *carotovora* did (Table 1). It also differed from *E. carotovora* subsp. *carotovora* for its ability to oxidise Tween 80 and lactic acid (Table 1). It was positive for catalase, Voges–Proskauer, methyl red and nitrate reduction but it was oxidase negative and it was not motile (Table 1). The Microlog system identified it as *E. carotovora* but the database did not contain any *E. cacticida* information. *E. cacticida* from blue agave was sensitive only to ceftriaxone (Table 1).

Circular colonies were observed on LB, CVP and PDP media. White and ivory colonies on LB and PDP media, respectively, but on CVP flat and rough colonies were produced at 28 and 43 °C. *E. cacticida* grew both on pectate media pH 5.0 and pH 7.0. Colonies were more apparent at pH 7.0 but did not form pits as did *E. carotovora* subsp. *carotovora* 71. *E. cacticida* grew on PDP at 43 °C. The cells were rod-shaped and measured ca. 0.9 μm by 1.1 μm.

16S rDNA gene sequence. The phylogenetic tree constructed using the 16S rDNA sequences, grouped together *P. agglomerans* I and III, but they were in a separate cluster from the other *Pantoea* spp. and *E. herbicola* strains (Figure 3). The *Pseudomonas* sp. was included in the same cluster with different Pseudomonands species, including *Burkholderia cepacia* and *Pseudomonas fluorescens* (Figure 3). *E. cacticida* was grouped together with *Pantoea ananas* and was completely separated from the *E. cacticida* and *E. carotovora* groups (Figure 3). *E. carotovora* is widely distributed in the phylogram, the subspecies *wasabiae* and *betavasculorum* were in the same group, while the subspecies *odorifera* formed a different group. *E. chrysanthemi* and *Pantoea* spp. formed homogeneous groups (Figure 3).

Intergenic transcribed spacer (ITS). After PCR amplification of the ITS, *P. agglomerans* I and III produced the fragments reported previously for *P. agglomerans* (Toth et al., 2001). The fragments of 650 and 490 bp (data not shown) were the same for both isolates and correlated with the size described for the species. *E. agglomerans* produced two bands of 450 and 600 bp as main fragments (data not shown), while *Pseudomonas* sp. did not amplify as expected. *E. cacticida* and *E. carotovora* subsp. *carotovora* 71 produced two different fragment sizes, both of them correlated with those described for each species. The primary fragments in *E. cacticida* were 550 and 580 bp (Figure 4A), although there was a light one of 660 bp instead of 740 bp. In *E. carotovora* subsp. *carotovora* 71, the main fragments were 540 and 575 bp. It also produced a light fragment of 660 bp (Figure 4A). After digestion of the ITS-PCR products of *E. cacticida* and *E. carotovora* subsp. *carotovora* 71, different banding patterns were generated (Figure 4B). *E. cacticida* produced two of the three fragments characteristic for the species, 355 and 565 bp, and a third one of 160 bp, (Figure 4B), while *E. carotovora* subsp. *carotovora* 71 produced the fragments 115, 180, 225 and 355 bp, correlating with previous reports (Toth et al., 2001) (Figure 4B).

Discussion

Many soft-rot diseases have been associated with *E. carotovora* which is characterised by the production of extracellular enzymes such as pectate lyases (Zucker et al., 1972; Kotoujansky, 1987; Daniels et al., 1988). Blue agave soft-rot symptoms were reproducible under greenhouse conditions and were similar to those observed in the field during initial stages of the disease.

In order to make an accurate diagnosis, it is important to identify and characterise the causal agents of a disease. Our results suggest that there is more than one bacterial species involved in blue agave soft-rot disease. These results were focused on the bacteria more frequently isolated from leaves showing soft-rot symptoms, suggesting they were the dominant species during the sampling dates.

In a previous report, Valenzuela-Zapata (1994) suggested *E. carotovora* was the causal agent of the soft-rot disease of *A. tequilana* leaves. However, based on Koch's postulates and on an extensive biochemical characterisation employing fatty acid analysis, carbon source utilisation profiles, physiologic properties, DNA sequences and ITS amplification and restriction banding pattern, the following species were identified: *E. cacticida*, two different strains of *P. agglomerans* and an isolate of *Pseudomonas* sp. *E. carotovora* was not isolated.

Analysis of fatty acids profiles is a frequently used method for characterisation and identification of

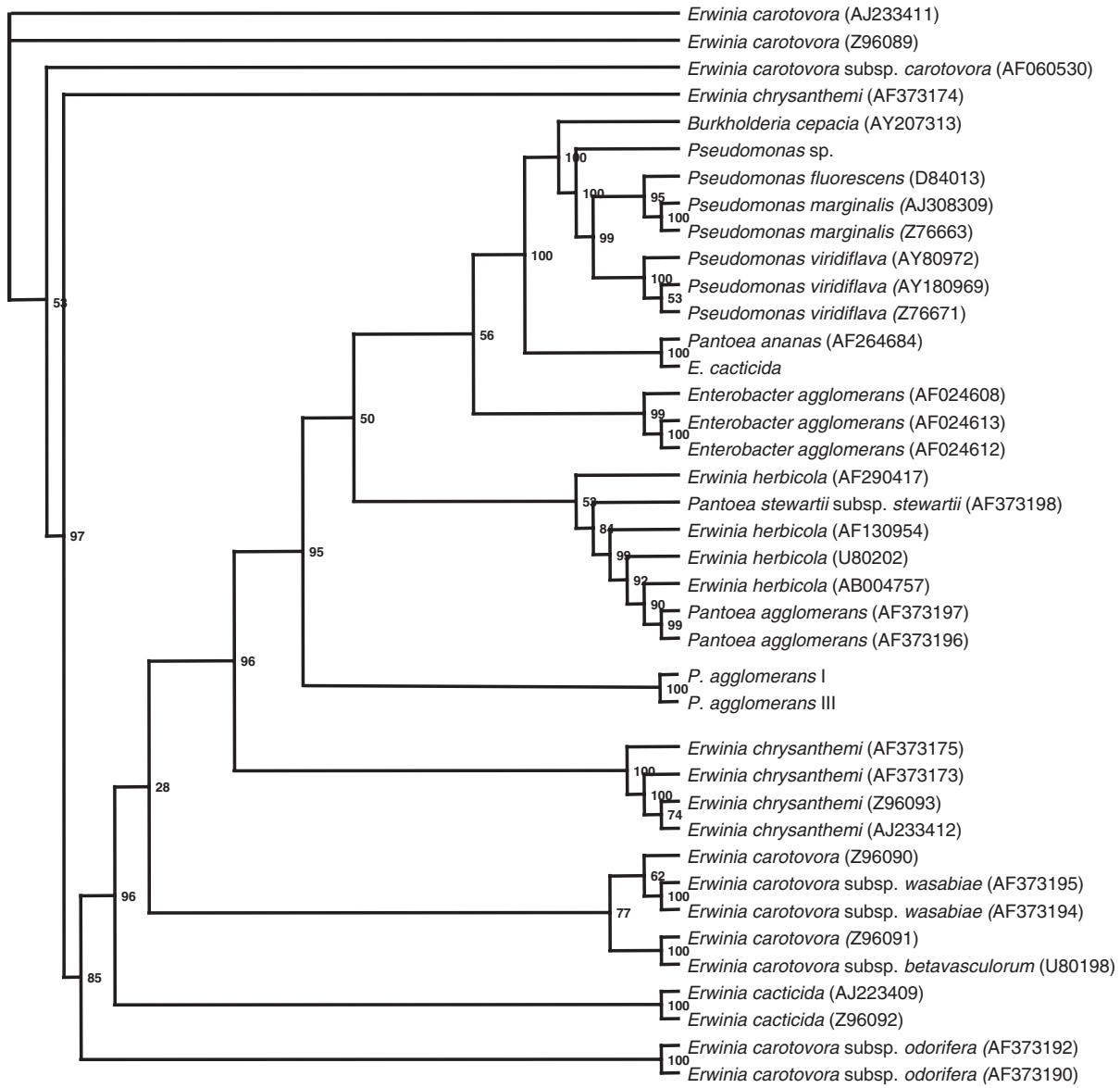

Figure 3. Phylogenetic tree showing the relationship of the isolated strains within a subset of closely related species. The tree is based on alignment of 16S rDNA sequences and was constructed using the Neighbour-Joining method. Stability of the tree was assessed by 1000 bootstrap replications with Felsenstein confidence limits. Accession numbers for type strains in GenBank are in parentheses.

bacterial isolates. The mean percentage of the major fatty acids present in the bacterial isolates described in this work did not correlate with previous reports for *E. carotovora*. (DeBoer and Sasser, 1986; Persson and Sletten, 1995). This is supported by the differences observed in the fatty acids ratios: 12 : 0/14 : 0; 16 : 0/12 : 0 and 16 : 1/18 : 1 (Table 3), which confirmed the identity of the isolates of *P. agglomerans* I and III, and *E. cacticida*. Seo (2002) reported the presence of the fatty acids 12 : 0; 16 : 0; 16 : 1cis 9 and 18 : 1cis11 in isolates of *E. carotovora* subsp. *carotovora*. The absence of 16 : 1cis 9 and 18 : 1cis 11 in the blue agave bacteria, corroborate they are different from *E. carotovora*. The low percentage of the fatty acid 13 : 0 and the absence of 17 : 1cis 9 in *E. cacticida*, correlate with Alcorn (1991), but not the presence of

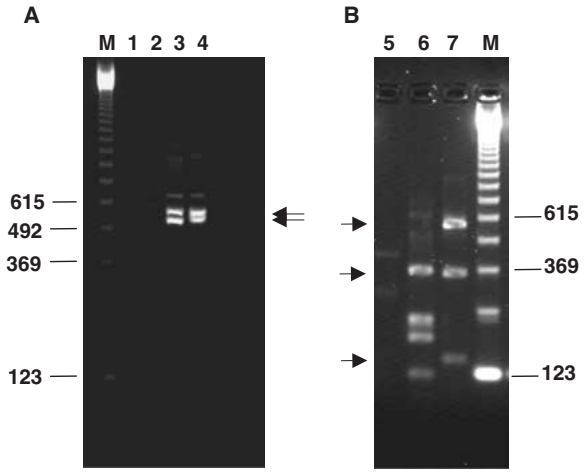

Figure 4. A. ITS-PCR amplification of *E. cacticida* isolated of blue agave leaves, and the type strains *E. carotovora* subsp. *carotovora* 71 and *E. agglomerans*, and B. digestion of PCR products with *Rsal* (ITS-RFLP). Lane 1, *E. agglomerans*; 2, *E. carotovora* subsp. *carotovora* 71; 3, *E. cacticida*; 4, control; 5, ITS-RFLP of *E. agglomerans*; 6, ITS-RFLP of *carotovora* subsp. *carotovora* 71 and 7, *E. cacticida* ITS-RFLP. M, 123 bp ladder (Invitrogen Co.). Arrows indicate the fragment size of ITS-PCR (550 and 580 bp) and ITS-RFLP (180, 355 and 565 bp) of *E. cacticida*.

the fatty acid 19 : 0 ciclo, suggesting it is a variant of the species.

The Biolog system easily identified type isolates, although previous reports (Jones et al., 1993; Toth et al., 1999) have found it has limited utility in identifying wild isolates. In this study, like in others (Lee et al., 1997; Gilho et al., 2002), we found it to be an effective method for metabolic characterisation of bacteria, even though the database does not consider *E. cacticida. Pseudomonas* sp. could not be classified at the species level. It did not use starch, trehalose, *m*-inositol and maltose as *P. mendocina*, *P. alcaligenes* or *P. pseudoalcaligenes* did (which were the species suggested on fatty acid analysis and ribosomal DNA partial sequence). Also it did not use glucose, malonate and saccharate as carbon source as *P. alcaligenes* and *P. pseudoalcaligenes* did (Holt et al., 1994). In *P. pseudoalcaligenes* there are two plant pathogenic subspecies: *P. pseudoalcaligenes* subsp. *konjaci* and *P. pseudoalcaligenes* subsp. *citrulli* (Holt et al., 1994). The latter is a pathogen of *Cucumis melo* and *Citrullus lanatus*, species in which it causes water-soaked lesions on the fruits (Isakeit et al., 1997; Pinto et al., 2000; Hopkins and Thompson, 2002).

The presence of two fatty acids (12 : OH and 10 : 3OH) common to other Pseudomonads (Rainey et al., 1994; Barnett et al., 1999), corroborates the identity of the genus of *Pseudomonas* sp. In different *Pseudomonas* species like in the Pseudomonads sp. from blue agave, the predominant fatty acids are 16 : 0, 16 : 1 and 18 : 1. Because this isolate shares similar characteristics with *P. pseudoalcaligenes* and the capability to growth at 41 °C, it is possible that *Pseudomonas* sp. (isolate VII), is involved in *A. tequilana* disease. *P. marginalis* produces pectin lyases that degrade the cell wall fragments generated by pectate lyases, therefore *Pseudomonas* sp. could participate in later stages of the disease after tissue maceration caused by other bacteria species. The phylogenetic tree confirmed that the *Pseudomonas* sp. is different from other soft-rot Pseudomonads. It is separated from them but close to *Burkholderia cepacia* that causes soft rot in onion. The production of yellow pigment, in KB medium, suggests it is a type of *Pseudomonas fluorescens*. However, further studies should be done to address its role during disease development and to determine its identity at the species level.

The genus *Erwinia* is very heterogeneous and has been re-classified in several groups on the basis of its biochemical characteristics and on 16S rDNA sequence homology (Hauben et al., 1995; Kwon et al., 1997; Spröer et al., 1999). The carotovora group consists of soft-rot bacteria while the herbicola group resembles some *Enterobacter* members, which were classified together with *Erwinia* sp. (Beji et al., 1988). The metabolic characteristics of *P. agglomerans* I and III were very similar to the herbicola–agglomerans complex, which includes bacteria that are both saprophytic and pathogenic on plants (Beji et al., 1988; Wells et al., 1987). The *Pantoea* genus was formed grouping together some strains of *E. agglomerans* and *E. herbicola* (Iimura and Hosono, 1996), therefore, *Pantoea* sp. shares some characteristics of both genera (*Erwinia* and *Enterobacter*). The carbon source utilisation of *P. agglomerans* I and III correlated with previous reports of *P. agglomerans* (Gavini et al., 1989; Holt et al., 1994, Iimura and Hosono, 1996). *Pantoea* spp. have been reported as plant pathogen. For example, *P. annanas* and *P. stewartii* are associated with leaf blotch disease of Sudangrass (Azad et al., 2000). Also, *Pantoea citrea* is related to pink disease in pineapple (Pujol and Kado, 2000), while *Pantoea ananatis* causes leaf blight and bulb decay of onion (Schwartz and Otto, 2000) and bacterial blight and dieback of *Eucalyptus* sp. (Coutinho et al., 2002). Another report indicated *Pantoea* sp. cause rotting of onion neck and between scales (Schwartz and Otto, 2000).

In succulent plants, *Enterobacter* species (synonym *Pantoea*) have been isolated from injured tissue of agria cactus (*Stenocereus gummosus*) (Foster and Fogleman, 1994). Cacti and agave plants grow on harsh environments, therefore, it was not surprising to find *P. agglomerans* (formerly *E. agglomerans*) isolates as inhabitants of *A. tequilana* leaves. These findings suggest *P. agglomerans* isolated from *A. tequilana* might participate at some stage in the development of the soft-rot disease. The bands observed in ITS-PCR correlated with those reported for *P. agglomerans* (Toth et al., 2001). The phylogenetic tree showed these strains are closely related to *Pantoea* spp., although they were in a different group due because *P. agglomerans* I and III matched some characteristics of both *Pantoea* (formerly *Enterobacter*) and *Erwinia* genera.

Some authors have associated the bacterium *E. cacticida* with soft rot of cacti (Alcorn et al., 1991; Foster and Fogleman, 1993). *E. cacticida* from *A. tequilana* demonstrated to cause soft-rot symptoms on both blue agave and cacti, and also was capable of producing pectate lyases. It is thought *P. agglomerans* I and III, and *E. cacticida* may have different isozymes of pectate lyase because they grow on different pectate media at different pH levels and temperatures, although further studies should be done; these characteristics suggest they are capable to degrade agave tissue as observed in the pathogenicity tests. The carbon source utilisation profile of *E. cacticida* correlated with results obtained previously (Alcorn et al., 1991; Beji et al., 1988; Hauben et al., 1998, Iimura and Hosono, 1996) for the *Erwinia* genus, except for L-arabinose in *E. cacticida*. Although the growth on cellobiose and raffinose did not correlate with *E. cacticida* previous reports, the utilisation of gentibiose and malonate, the growth on PDP at 43 °C and being Voges–Proskauer positive, correlated with *E. cacticida* (Alcorn et al., 1991; Foster and Fogleman, 1994). *Erwinia chrysanthemi*, is able to grow at high temperatures (35–39 °C) and it is considered to cause soft-rot symptoms in tropical and subtropical regions (Farrar et al., 2000) but isolate IX correlates more with *E. cacticida* previous description. It also differs from *E. chrysanthemi* on its ability to oxidise D-trehalose and its inability to utilise D-sorbitol (Table 1). *E. cacticida* is considered to be more related to *E. carotovora* than to *E. chrysanthemi* (Toth et al., 2001). This affinity shows the big phenotypic and genotypic similarity (97.3%) between the two species (*E. carotovora* and *E. cacticida*) (Hauben et al., 1998), although *E. cacticida* formed a separate group. *E. carotovora* is very heterogeneous but *E. chrysanthemi* has shown to be genetic and phenotypically homogeneous (Fessehaie et al., 2002). The tree generated based on 16S rDNA sequences demonstrated that *E. chrysanthemi* formed a single group, while *E. carotovora* is in 5 different groups (Figure 3). This demonstrates the complexity of the genus. *E. cacticida* did not group together with none of them but with *P. ananas*. This could be because of the small fragment (615 bp) compared with the rest of the strains (1500 bp). It also showed that *E. cacticida* from *A. tequilana* differs from the other soft-rot *Erwinia*s (*E. chrysanthemi*, *E. carotovora* and *E. cacticida*): because *E. cacticida* did not match exactly either the fatty acid profile or the metabolic characteristics, it was considered as an atypical strain but 16S rDNA sequences and ITS-PCR corroborate the *E. cacticida* identification. It produced the two ITS-PCR primary bands reported for the species (550 and 580 bp) (Toth et al., 2001). The presence of the 660 bp fragment supports *E. cacticida* as a variant strain of the species. It also produced the characteristic banding pattern of ITS-RFLP, which shows that genotypically it is a strain of *E. cacticida* but with different phenotype.

Erwinia cacticida is considered the most pathogenic bacterium to *A. tequilana* on the basis of the rapid decline produced on the different hosts tested (blue agave, potato, cucumber, tomato and cactus plant). It might be the main pathogen in the disease followed by *P. agglomerans* I and III. *E. cacticida*, despite having biochemical similarities with the type strain *E. cacticida*, does not match them exactly; therefore, we propose it as a new variant of *E. cacticida* found to cause soft rot on leaves of *A. tequilana*.

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