ELSEVIER

System. Appl. Microbiol. 27, 27–35 (2004)
http://www.elsevier-deutschland.de/syapm

SYSTEMATIC AND APPLIED MICROBIOLOGY

Klebsiella variicola, A Novel Species with Clinical and Plant-Associated Isolates

Mónica Rosenblueth[1], Lucía Martínez[1], Jesús Silva[2], and Esperanza Martínez-Romero[1]

[1] Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernavaca, Morelos, México
[2] Instituto Nacional de Salud Pública, Cuernavaca, Morelos, México

Received: October 10, 2003

Summary

A new *Klebsiella* species, *K. variicola*, is proposed on the basis of total DNA-DNA hybridization, on the monophyly observed in the phylogenetic analysis derived from the sequences of *rpoB, gyrA, mdh, infB, phoE* and *nifH* genes and on distinct phenotypic traits. The bacteria from this new species seem to be genetically isolated from *K. pneumoniae* strains, do not ferment adonitol and were obtained from plants (such as banana, rice, sugar cane and maize) and hospitals. The type strain is F2R9^T (= ATCC BAA-830^T = CFNE 2004^T).

Key words: Enterobacteria – nitrogen fixation – *Klebsiella* – taxonomy – novel species

Introduction

With increasing frequency, bacteria of the genus *Klebsiella* have been found associated with hospital infections and account for around 8% of the total infections of this type [41]. Based on *rpoB* gene sequences a proposal emerged to transfer some of the *Klebsiella* species, namely *K. planticola, K. terrigena* and *K. ornithinolytica*, to the new genus *Raoultella* [12]. Most of the clinical isolates belong to *K. pneumoniae, K. oxytoca* and *K. granulomatis* (the presumptive agent of donovanosis, a genital ulceration [7]) but a minor proportion correspond to *Raoultella planticola* (8–19% of the clinical isolates) and to *R. terrigena* (0.4% of the clinical isolates). Clinical and environmental *K. pneumoniae* isolates may be distinguished based on virulence [19] but in other cases, environmental *K. pneumoniae* isolates were as virulent as the clinical isolates [32, 39]. *K. oxytoca* and *R. planticola* from fresh waters had less virulence traits [40].

R. planticola and *R. ornithinolytica* that produce large amounts of histamine are involved in food poisoning by fish [26]. *Klebsiella* spp. have been isolated from insects [23] and mammalian guts [16] and *K. pneumoniae* and *R. planticola* strains have been isolated from diverse plants [2, 20] including rice [29] and maize [39]. Pathogenic *Klebsiella* have been found to successfully colonize potato and lettuce [27] and plants may be considered to be reservoirs of human opportunistic *Klebsiella*. Inside plants, bacteria encounter new niches where adapted clones may be selected, and some endophytic bacteria could represent novel species. Endophytes must have mechanisms to colonize internal plant tissues [28] and to survive plant defense reactions, as well as to adequatedly use plant nutrients without causing harm to the plant. In maize plants, *Klebsiella pneumoniae* isolates colonize internal tissues and fix nitrogen if provided with an additional carbon source [9]. In wheat, some maize borne strains of *Klebsiella pneumoniae* fixed nitrogen but clinical isolates were less efficient in colonizing wheat [11]. Maize field inoculation trials with maize *K. pneumoniae* bacteria have been performed in the USA and better crop yields were recorded (E. Triplett, personal communication). Maize *Klebsiella pneumoniae* isolates had less virulence determinants than clinical isolates [11]. The search for genetic virulence determinants has been approached by comparing bacteria within the same species that have contrasting phenotypes (pathogenic and non-pathogenic); to do this, the determination of biological coherent groups (species) is indispensable. Interspecific substraction hybridization experiments would likely result in picking up metabolic (housekeeping) diverging genes that differ among the species tested but may not be related to pathogenesis.

By total DNA-DNA hybridization [13, 25] at least 5 groups were reported, including *K. pneumoniae* with its three subspecies (*ozaenae, rhinoscleromatis* and *pneumoniae*), *K. oxytoca, R. planticola, R. terrigena and K. mobilis* (also considered *Enterobacter aerogenes*). The relative genetic relationships of some *Klebsiella* species and

0723-2020/04/27/01-27 $ 30.00/0

other enterobacteria have been established from the analysis of several gene sequences [4, 7, 12, 22 , 36] revealing that the genus *Klebsiella* was polyphyletic and that *R. planticola* and *R. ornithinolytica* are closely related.

One characteristic of *Klebsiella* spp. is the presence of different alleles of a β-lactamase gene [14, 17] in the chromosome. Some extended-spectrum β-lactamases (ESBLs) that are able to hydrolyze oxyimino-β-lactams are encoded in plasmids [6, 46].

We isolated and characterized *Klebsiella* spp. related to *K. pneumoniae* [30] from inside banana tissues, leaves and stems and also from banana plantlets derived from tissue culture. The large genetic distance determined from alloenzyme analyses and the low DNA-DNA relatedness between *K. pneumoniae* type strain ATCC 13883^T and some of the banana isolates were suggestive of their belonging to a novel species. In this work we present polyphasic evidence [51] to show that these bacteria correspond to a new *Klebsiella* species. Our analysis included 131 clinical isolates from Mexico to ascertain if some of them were also members of this species.

Materials and Methods

Bacterial strains and media

One hundred thirty one clinical isolates of *Klebsiella* were included in this study, these were obtained from blood (63 strains), urine (24 strains), catheters (15 strains), secretions (8 strains), spinal liquid (3 strains) and other sites (18 strains). Collection years were 1990–1993 (22 strains) and 1996–2001 (109 strains) from 9 different Mexican hospitals. Identification was performed with the API 20E system (bioMérieux sa, Marcy l'Etoile, France). Clinical as well as plant isolates were from geographically separated regions.

Table 1. PCR primers and conditions.

Genes	Primers	Annealing temperature (°C)	Product (bp)
rpoB	CM31b CM7 [36]	65	1089
gyrA	*gyrA*-A *gyrA*-C [4]	65	416
nifH	*polF* *polR* [42]	56	360
infB	*infB*867F (ACA-AAG-TTA-TCC-TGC-GTC-GTG) *infB*1819R (TGA-ACT-TCG-CCT-TCG-GTC-ATG)	59	973
phoE	*phoE*840.1 *phoE*840.2 [7]	55	840
mdh	*mdh*130F (CCC-AAC-TCG-CTT-CAG-GTT-CAG) *mdh*867R (CCG-TTT-TTC-CCC-AGC-AGC-AG)	59	757

Rice isolates were obtained from macerated washed roots from rice cultivar A98, other plant isolates were from Martínez et al. [31].

All clinical and plant *Klebsiella* isolates were routinely grown on MacConkey plates and maintained in LB medium [35] with 15% of glycerol at –70 °C. Adonitol (also known as ribitol), and rhamnose fermentation were tested at 6.25 g l^{-1} in the minimal medium described [56] excluding sodium deoxycholate and with bromothymol blue as indicator. The reference strains used were: *K. pneumoniae* subsp. *pneumoniae* ATCC 13883^T, *R. planticola* ATCC 33531^T, *R. terrigena* ATCC 33257^T and *K. oxytoca* ATCC 13182^T. Antibiotic resistance was tested in LB medium with 50 mg l^{-1} of carbenicillin or ampicillin; 20 mg l^{-1} nalidixic acid; spectinomycin 250 mgl^{-1} or gentamicin 10 mg l^{-1}. Other media used were PY [50] or YM [52], with 15 g agar per liter. Motility was determined on 0.3% agar in MacConkey or LB media in plates.

PCR, sequencing and sequence analysis

For PCR amplification we used the primers shown in Table 1. The primers used to amplify *infB* and *mdh* were designed based on the alignment of these *K. pneumoniae* genes with those of other bacteria available in the public sequence database. All PCR amplification assays were performed in a 50 µl reaction volume, containing approximately 5 ng DNA with 10 pmol of each primer, 200 µM dNTPs, 1.5 mM of $MgCl_2$ and 1 U Taq DNA polymerase (Boehringer Mannheim). The reaction mixture was incubated for 4 min at 94 °C, and then subjected to 35 cycles consisting of 1 min at 94 °C, 1 min at the specific annealing temperature of each primer set (see Table 1) and 1 min at 72 °C, with a final step of 5 min at 72 °C. PCR products were sequenced using an ABI 310 automated sequencer (Applied Biosystems) following the manufacturer's recommendations.

The nucleotide sequences were aligned using ClustalW. Aligned sequences were analyzed using the Molecular Evolutionary Genetic Analysis (MEGA) Package version 1.01 to produce Neighbour-Joining under Jukes-Cantor, Kimura 2-parameters models as well as Minimum Evolution and unweighted Maximum Parsimony trees.

DNA-DNA hybridization

Klebsiella strains were grown overnight in 4 ml liquid LB medium and DNA was extracted from 1 ml cultures with Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham, USA). DNAs were digested with *Bam*H1, transferred to nylon filters and hybridized to the total DNA from the banana isolate F2R9^T or *K. pneumoniae* ATCC 13883^T, *R. planticola* ATCC 33531^T or *R. oxytoca* ATCC 13182^T each labelled with ^{32}P-CTP using RediPrime (Amersham). Hybridization was performed at 60 °C, washings were with 2 × SSC at 60 °C for 30 min and twice with 1 × SSC for 30 min. Individual lanes were cut and counted in scintillation liquid in a Beckman scintillation counter. Percent hybridization for each strain was estimated in reference to the total homologous hybridization as average of 2 or 3 hybridization (SD indicated in these cases) assays using the conditions employed when we described new and well recognized *Rhizobium* species [32, 50, 54, 55] or *Gluconacetobacter* spp. [15].

Nitrogen fixation determination

Acetylene reduction activity (ARA) was used to identify nitrogen fixing bacteria from *Klebsiella* strains grown in modified acetic LGI [8] with 2 g sucrose l^{-1}, pH 6.5 with semisolid agar (1.8 g l^{-1}), grown for 3 or 6 days and then injected for 3 or 12 hrs with acetylene. Ethylene was detected in duplicate samples by gas chromatography as described [44].

Plasmid patterns

Klebsiella strains were grown from fresh cultures on MacConkey plates in 2 ml LB liquid medium for about 3 hrs until they attained an O.D. at 600 nm of 0.4–0.6. For direct lysis 0.2 ml of cultures were placed in modified Eckhardt gels with agarose-SDS as described [24]. Gels with the plasmid profiles were blotted onto nylon filters and were used in ^{32}P radioactive Southern blot hybridizations with *nifH* product (as a probe) obtained by PCR from *Klebsiella* strain 6A2 (see below). Plasmid hybridization to the *nifH* gene was used to determine if *nifH* genes were plasmid borne and was performed following the procedures described [53].

Results and Discussion

Klebsiella isolates from banana plants had 16S rRNA gene sequences similar (96–97% identity) to those of *K. pneumoniae* [31]. They were characterized by their electrophoretic patterns of metabolic enzymes and some of the isolates from banana plants were found to group with maize and sugar cane-borne strains constituting an independent cluster at a genetic distance >0.5 from the reference strains of *K. pneumoniae*, *K. oxytoca*, *R. planticola* and *R. terrigena* [31]. Genetic distances larger than 0.5 have been found among different bacterial species [37, 45]. Total DNA-DNA hybridization resulted only in low levels of hybridization of *K. pneumoniae* with the banana isolates tested [31]. Taken together these results suggest that these isolates may correspond to a new species, and here we present data to endorse a novel *Klebsiella* species.

Phenotypic selection of strains

Klebsiella strains are recognized by their characteristic yellow colonies on acidic Koser citrate medium [5] and by being non motile [18]. All 131 *Klebsiella* clinical and the plant isolates tested formed yellow colonies on acidic Koser citrate medium and were non-motile. Adonitol is generally utilized and fermented by *Klebsiella* [18] but the three banana isolates F2R9^T, 6A2 and VI, strain T29A from sugar cane, strain 3 from maize, strain CFNE 2006 from rice and 7 of the 131 clinical isolates (5.3% of the total) did not ferment adonitol. All adonitol negative strains were selected (Table 2). One adonitol positive clinical strain (910) was also included in the sequencing assays, as well as strain B5R5 [31], an *Enterobacter* banana isolate. The selected isolates were used in the comparison of the nucleotide sequences of the genes that have been used in other *Klebsiella* species studies [4, 7, 12], for DNA-DNA hybridizations and for phenotypic characterization.

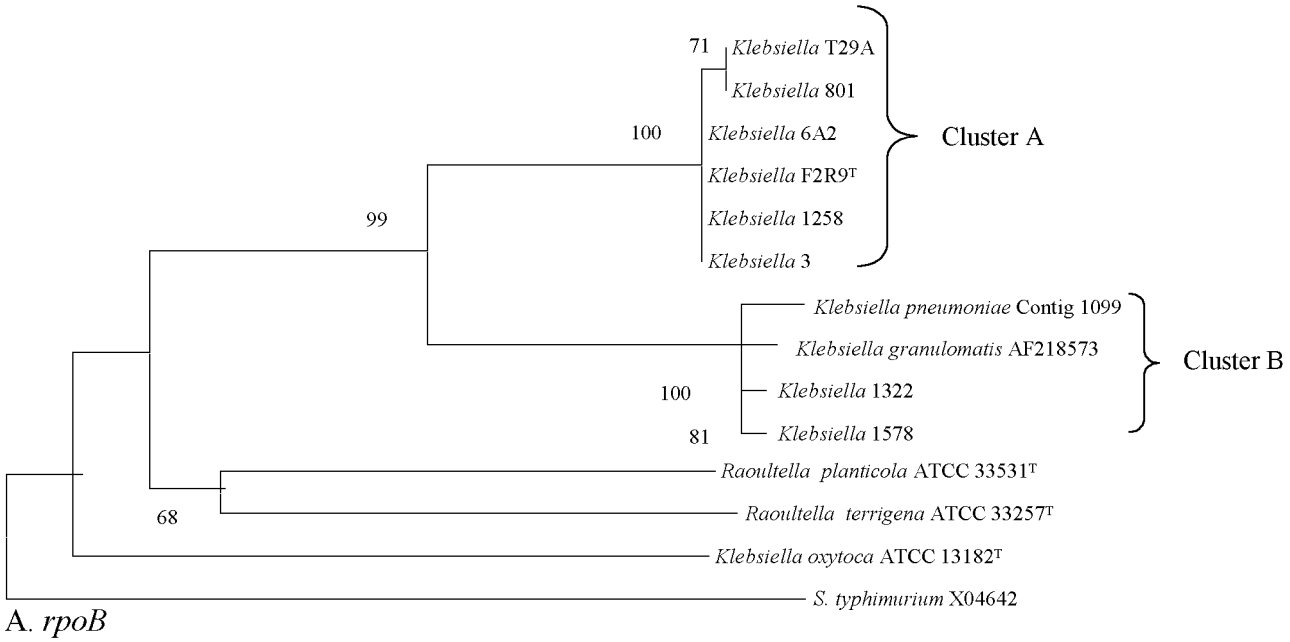
Gene sequences and phylogenetic analysis

The analysis derived from *rpo* sequence was used to propose *Raoultella* as a genus with 6% *rpoB* sequence dissimilarity considered as a reasonable limit to differentiate genera [12]. *rpoB* gene encodes the β-subunit of the RNA polymerase. A 1024 bp sequence of *rpoB* was determined for *Klebsiella* plant isolates T29A, 6A2, F2R9^T and 3, and from the type strains of *R. terrigena*, *R. planticola*, *K. oxytoca*, *K. granulomatis* and *Klebsiella* clinical isolates 801 and 1258; other sequences were obtained from data bases (Fig. 1). Banana isolates and clinical isolates had highly similar *rpoB* alleles. A well defined group (A), supported by high bootstrap values, was formed in the dendrogram reconstructed with these data (Fig. 1A). For other isolates (1109, 1171, CFNE 2006) and 910 (an adonitol positive strain) only a shorter sequence of *rpoB* (460 bp corresponding to bases 1975–2434 of the complete *Escherichia coli rpoB* gene) was considered in the analysis revealing that these isolates, excluding 910, belonged to cluster A as well (not shown and Table 2).

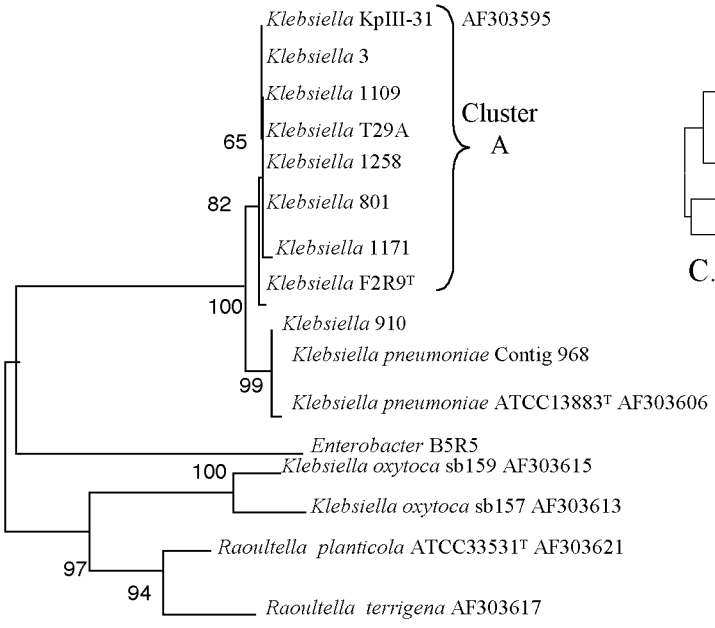
Table 2. *Klebsiella* isolates selected as incapable of fermenting adonitol. Nucleotide sequence cluster was based on *rpoB* sequence.

Strain	Source	Nucleotide sequence cluster	ARA[a] (nmol/hr/vial)	Rhamnose Utilization	Antibiotic[g]	
					Sp	Gm
F2R9^T	Banana root. Colima	A	1.9	+	S	S
T29A	Sugar cane stem. Sinaloa	A	237.5	+	S	S
801	Blood. Del Niño H[b], Villahermosa, Tabasco	A	417.4	+	S	R
1109	Blood. Niño Morelense H[b], Cuernavaca, Mor.	A	344.2	+	S	S
1171	Catheter. Niño Morelense H[b]	A	369.0	+	S	S
1258	Blood. Infantil de México H[b], Mexico City	A	344.4	+	S	R
6A2	Banana leaves, TC[c]. Colima	A	290.9	–	S	S
VI	Banana stem. Morelos	NS[e]	150.0	–	S	S
3	Maize shoots. Oaxaca	A	341.6	–	S	S
CFNE 2006	Rice roots. Morelos	A	130.0	+	S	S
806	Blood. Del Niño H[b], Villahermosa, Tabasco	A	435.7	–	S	R
1322	Sf[d]. Infantil de México H[b]	B[f]	0	+	R	R
1578	Blood. Pediatría S.XXI IMSS H[b], Mexico City	B[f]	0	+	R	R

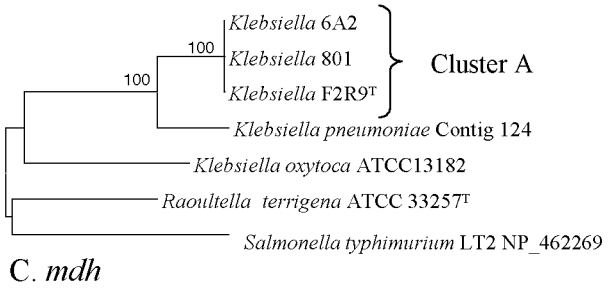
[a] Acetylene reduction assay, [b] Hospital, [c] Tissue culture, [d] Spinal fluid, [e] Not sequenced, [f] Related to *K. pneumoniae* (see Fig. 1). [g] Antibiotics: Sp = spectinomycin 250 mg l^{-1} or Gm = gentamicin 10 mg l^{-1}, S = susceptible, R = resistant.



A. *rpoB*

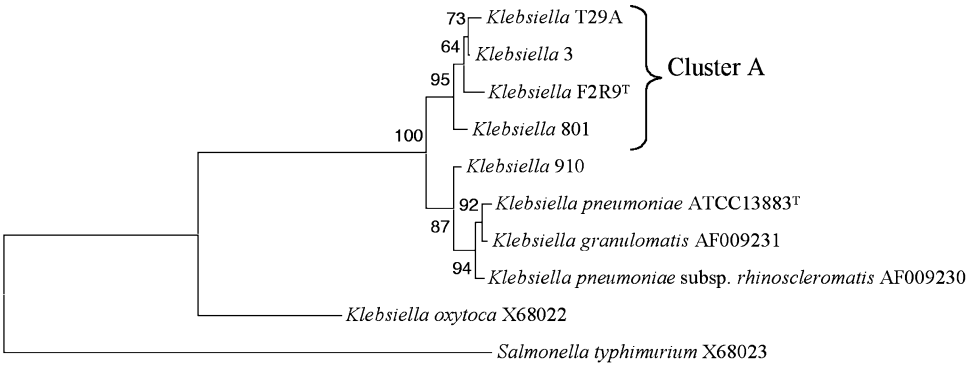


B. *gyrA*

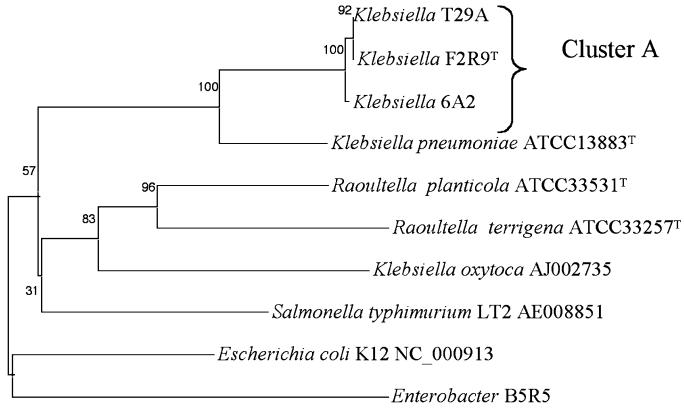


C. *mdh*

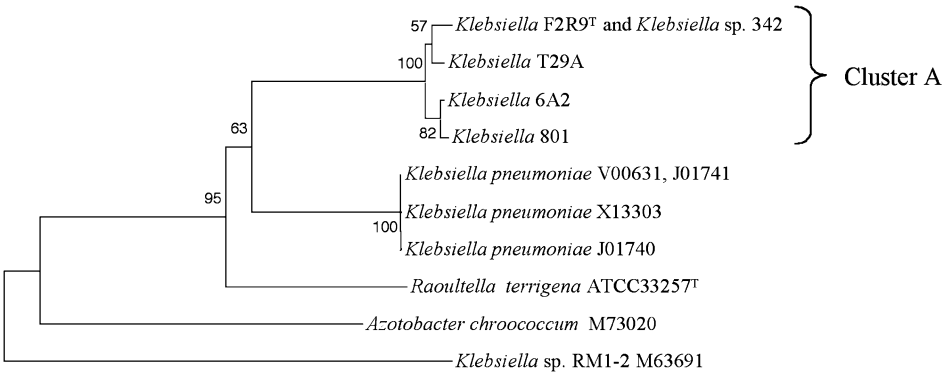
Fig. 1. Phylogenetic trees (Kimura 2-parameters) of different partial gene sequences using A) *rpoB* (1024 bp analyzed); B) *gyrA* (395 bp); C) *mdh* (669 bp); D) *phoE* (741 bp); E) *infB* (906 bp); F) *nifH* (312 bp). The following gene sequences were deposited in GenBank under the accession numbers given in parenthesis: *rpoB* from strains T29A (AY367353), 801 (AY367354), 6A2 (AY367355), F2R9^T (AY367356), 1258 (AY367357), 3 (AY367358), 1109 (AY367364), 1171 (AY367365), CFNE 2006 (AY438584), 1322 (AY367359), 1578 (AY367360), 910 (AY367366), *Raoultella planticola* ATCC 33531^T (AY367361), *Raoultella terrigena* ATCC 33257^T (AY367362), *Klebsiella oxytoca* ATCC 13182^T (AY367363) and *Enterobacter* sp. B5R5 (AY367367); *gyrA* from strains 3 (AY367368),



D. *phoE*



E. *infB*



E. *nifH*

1109 (AY367369), T29A (AY367370), 1258 (AY367371), 801 (AY367372), 1171 (AY367373), F2R9^T (AY367374), 910 (AY367375) and *Enterobacter* sp. B5R5 (AY367376); *mdh* from 6A2 (AY367377), 801 (AY367378), F2R9^T (AY367379), *Klebsiella oxytoca* ATCC 13182^T (AY367380) and *Raoultella terrigena* ATCC 33257^T (AY367381); *phoE* from strains T29A (AY367382), 3 (AY367383), F2R9^T (AY367384), 801 (AY367385) and 910 (AY367386); *infB* from strains T29A (AY367387), F2R9^T (AY367388), 6A2 (AY367389), *Raoultella planticola* ATCC 33531^T (AY367390) and *Raoultella terrigena* ATCC 33257^T (AY367391); *nifH* from strains F2R9^T (AY367392), T29A (AY367393), 6A2 (AY367394), 801 (AY367395) and *Raoultella terrigena* ATCC 33257^T (AY367396).

The independent cluster A seems to branch together with *K. pneumoniae* from a common ancestor. Cluster A was also obtained with the *gyrA* (Fig. 1B), *mdh* (Fig. 1C), *phoE* (Fig. 1D), *infB* (Fig. 1E) and *nifH* (Fig. 1F) gene sequence analyses. *gyrA* codes for subunit A of DNA gyrase and its phylogenies correspond to those derived by *parC* (coding for subunit C of topoisomerase IV) [4]. *K. pneumoniae gyrA* gene is expressed during mice infection [30]. *mdh* codes for malate dehydrogenase, which is currently used in Multilocus Sequence Typing analysis [38], *phoE* codes for a phosphate transporter [7] and *infB* for the initiation factor 2 [22]. *nifH* codes for the nitrogenase reductase of the nitrogenase enzyme that converts atmospheric N to ammonium and the gene is part of a *nif-fix* gene cluster [1] in the *Klebsiella* chromosome.

Phylogenetic topologies were equivalent with all the reconstruction methods used (Neighbour Joining: Jukes-Cantor and Kimura 2-parameters; Minimum Evolution and Maximum Parsimony trees). Kimura 2-parameter trees are shown in Fig. 1. The phylogenies derived from the gene sequences analyzed (Fig. 1) showed that cluster A conforms a highly supported monophyletic lineage that may deserve a new species name within the genus *Klebsiella*. The fact that cluster A was maintained in all dendrograms derived from the different genes analyzed (thus not sharing alleles with *K. pneumoniae*, *K. oxytoca* nor with *K. granulomatis*) indicates that there is not extensive recombination of cluster A members with the neighbor species *K. pneumonia* nor with *K. granulomatis* or *K. oxytoca.* This supports their genetic isolation, a requisite for an independent species.

Our data support (Fig. 1) the suggestion that *K. granulomatis* may be a subspecies of *K. pneumoniae* [7].

Species delineation is more robust when some gene sequence data of representatives of the species are also available [48]. In the era of phylogenetic markers, species should be described mainly on these bases. The number of genes that need to be analyzed to generate a meaningful assignment may vary from species to species depending on the level of existing recombination and the sequence of 5 genes was considered sufficient [48]. The recombination rates in *Klebsiella* spp. are unknown but on the basis of alloenzyme patterns, the genetic structure of *K. pneumoniae* and *K. oxytoca* seems to be clonal [10, 17]. If this is the case, few chromosomal taxonomic markers may provide a good sample of the genome. It has been observed that prokaryotic genes involved in transcription and translation are less prone to lateral transfer than housekeeping genes [43], and for this reason we used the *gyrA* and *rpoB* genes as phylogenetic markers. Nevertheless, clade A was resolved with the housekeeping genes *mdh*, *phoE* and *infB* as well as with *gyrA* and *rpoB*. In addition, the genes that we analyzed map in different chromosomal positions on the *E. coli* genome, and if this holds for *Klebsiella*, then these genes may provide an adequate sample of the basic genomic frame that seems to constitute the common basic core of a bacterial species. We consider that 16S rRNA genes are not very convenient to define enterobacterial species since they are too conserved and they may be subject to recombination and gene conversion [21]. In *Klebsiella* banana isolate 6A2 we found 7 16S rRNA gene copies (not shown).

DNA-DNA hybridization

DNA-DNA hybridization is used as a primary tool to define a species and a 70% limit has been recognized

Table 3. DNA-DNA relatedness evaluated as% of hybridization to the homologous strains.

Strains tested		Probes			
		F2R9[Ta]	*K. pneumoniae*[a] ATCC 13883[T]	*R. planticola*[a] ATCC 33257[T]	*K. oxytoca*[a] ATCC 13182[T]
F2R9[T]	Cluster A	100.0	19.5 ± 1.3[b]	8.3	19.4
T29A		100.0	23.1 ± 1.0	7.4	
VI		–	25.4 ± 1.9	7.0	20.0
3		–	18.8 ± 1.2	11.0	
6A2		90.4	–	7.0	20.0
801		92.8	–	–	
1258		91.3	–	–	
1171		97.2	–	–	
CFNE 2006		98.0			
910		64.1	–	–	
1322		59.7	–	–	
K. pneumoniae ATCC 13883[T]		20.82 ± 1.5	100	–	
R. planticola ATCC 33531[T]		8.3	16.0	100.0	
R. terrigena ATCC 33257[T]		7.5	–	14.0	
K. oxytoca ATCC 13182[T]					100.0

– = not determined

[a] ^{32}P labelled total DNA as probes in Southern type hybridization assays

[b] SD

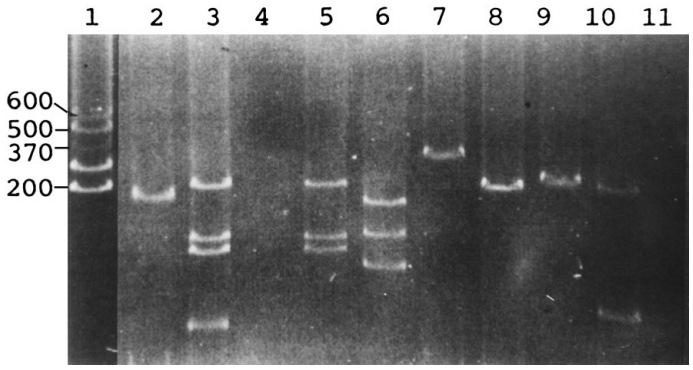


Fig. 2. Plasmid patterns visualized after agarose gel electrophoresis using a modified Eckhardt procedure [24]. Lanes 1) *Rhizobium etli* CFN42 as a marker of plasmid sizes; 2) *K. pneumoniae* adonitol negative isolate 1322; *K. variicola* isolates: 3) 3; 4) VI; 5) 6A2; 6) 1258; 7) 1171; 8) 1109; 9) 801; 10) T29A; 11) F2R9^T.

[49]. The results of DNA-DNA hybridization are presented in Table 3 and confirm the low DNA-DNA hybridization (20–23%) reported previously of *K. pneumoniae* ATCC 13883^T with F2R9^T, VI or 6A2 [31]. On average, *K. pneumoniae* and strains 3, F2R9^T, and T29A and VI had 22% DNA-DNA relatedness. With *K. oxytoca* total DNA hybridization was around 20% with F2R9, 6A2 and VI. Hybridization to total DNA from F2R9^T strain showed that cluster A strains T29A, 6A2, 801, 1258, and 1171 exhibited a higher DNA-DNA hybridization (more than 90%) than strains 910 and 1322 that do not correspond to cluster A. We could not test hybridization to *K. granulomatis* because it is not culturable in standard microbiological media and it has no type strain defined. Similarly no DNA-DNA hybridization of *K. pneumoniae* and *K. granulomatis* has been determined [7].

Additional phenotypic and genotypic characteristics

Rhamnose is commonly found as a component of dicotyledonous plant cell walls in the polysaccharide rhamnogalacturonan [34]. Different rhamnose utilization capacities were detected among cluster A isolates (Table 2).

Nitrogen fixation was found in 13 out of 15 of the adonitol negative isolates and in all of group A isolates but not in the two isolates not included in cluster A (Table 2). Most adonitol negative *Klebsiella* isolates had plasmids (Fig. 2). One of them was around 400 kb but most were about 200 kb or lower. No plasmids could be observed in F2R9^T or VI. None of *Klebsiella* plasmids hybridized to *nif* genes but hybridization was observed with the symbiotic plasmid (370 kb) of *Rhizobium etli* CFN42 used as a control (not shown). This indicated that the *nifH* genes of group A strains are chromosomally located as is the case in *K. pneumoniae* and other enterobacteria except for *Rahnella aquatilis* [3] and *Enterobacter agglomerans* [47].

The adonitol negative strains not belonging to cluster A were resistant to spectinomycin 250 mgl^{-1} and gentamicin 10 mgl^{-1}, while cluster A strains were susceptible to spectinomycin and most were susceptible to gentamicin.

Reclassification of reported strains

The new species seems to include *K. pneumoniae* cluster III isolates defined by Brisse and Verhoef [4]. This is based on the fact that cluster III isolates were also adonitol negative and have similar *gyrA* sequences (Fig. 1B, see strain KpIII-31 from Brisse and Verhoef, [4]). These isolates were all clinical, from a wide geographical origin in Europe and from Turkey and represented around 10% of the *K. pneumoniae* isolates reported [4]. Brisse and Verhoef [4] described a more closely related cluster to *K. pneumonia* (cluster II) and found that some of the isolates were adonitol negative as well. We do not know if any of our isolates correspond to this group but the candidates are the clinical isolates 1322 and 1578.

The new species should also include 18 other banana isolates that had the same electrophoretic type, defined by the mobility of 10 metabolic enzymes, as 6A2 and VI [31] and it may also include the maize isolate of *K. pneumoniae* strain 342 [39], since its *nifH* sequence is identical to that of F2R9 (Fig. 1F), although *K. pneumoniae* 342 has a high percent of DNA-DNA hybridization to the *K. pneumoniae* type strain but lower than 70% to other clinical *K. pneumoniae* isolates [11] and sequence analysis of other genes of strain 342 would be required to confirm its taxonomic status. It also remains to be established if some other plant isolates such as those from rice reported as *Klebsiella* [29] are members of this new species as well.

Characteristics of the novel species ***Klebsiella variicola* sp. nov.**

(va.ri.i' co.la. L. adj. varius different, differing, various, L. suffix n. -cola inhabitant , N.L. fem./masc. n. variicola inhabitant of different places):

The isolates of this new species are Gram negative rods. They grow in acidic Koser citrate, in MacConkey, in LB, in YM and in PY media. They form colonies on MacConkey medium within one day. They are nitrogen fixing bacteria. They do not ferment adonitol (a distinctive characteristic from *K. pneumoniae*) but many ferment

rhamnose. They grow at 37 °C and at pH 5.6–7. They are abundant in plants [31] and represent less than 10% of the clinical *Klebsiella* isolates previously considered as *K. pneumoniae*.

They correspond to the isolates that are considered within the sequencing cluster A reported here and are distinguished from *K. pneumoniae* and other *Klebsiella* species by their sequences of *rpoB, gyrA, mdh, phoE, infB* and *nifH* genes and by their low levels of DNA-DNA hybridization. The pathogenicity of the plant isolates from *K. variicola* has not been determined. As other strains of *Klebsiella pneumoniae* and *K. oxytoca*, *K. variicola* strains are susceptible to nalidixic acid but naturally resistant to ampicillin and carbenicillin but not in the presence of clavulanic acid that inhibits penicillinase. Most of them are sensitive to gentamicin 10 mgl^{-1} and they are sensitive to spectinomycin 250 mgl^{-1}.

Description of the type strain F2R9^T

The type strain F2R9^T has the characteristics of the species. It has been deposited as ATCC BAA-830, DSM 15968 and CFNE 2004. It is freely available from the CFN (Centro de Investigación sobre Fijación de Nitrógeno, Ap. postal 565-A, Cuernavaca, Mor. México) collection.

Acknowledgements

To J. Martínez Romero, A. Sánchez and M. A. Rogel for technical help, to R. E. Gómez Barreto for sequencing, to M. Dunn, C. Silva and P. Vinuesa for reading the manuscript. To Dr. Michael Molitor from Diagnostic, Germany, to Dr. Hans G. Trüper for their kind assistance, to Jorge Soto for providing rice isolates. Partial support was from CONACyT 25075-B and 30938. LM had a Ph. D. fellowship from CONACyT and DGAPA from UNAM.

References

1. Arnold, W., Rump, A., Klipp, W., Priefer, U. B., Pühler, A.: Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J. Mol. Biol. 203, 715–738 (1988).
2. Bagley, S. T., Seidler, R. J., Brenner, D. J.: *Klebsiella planticola* sp. nov.: a new species of Enterobacteriaceae found primarily in nonclinical environments. Curr. Microbiol. 6, 105–109 (1981).
3. Berge, O., Heulin, T., Achouak, W.: *Rahnella aquatilis*, a nitrogen-fixing enteric bacterium associated with the rhizosphere of wheat and maize. Can. J. Microbiol. 36, 195–203 (1990).
4. Brisse, S., Verhoef, J.: Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* genes sequencing and automated ribotyping. Int. J. Syst. Evol. Microbiol. 51, 915–924 (2001).
5. Bruce, S. K., Schick, D. G., Tanaka, L., Jiménez, E. M., Montgomerie, J. Z.: Selective medium for isolation of *Klebsiella pneumoniae*. J. Clinical. Microbiol. 13, 1114–1116 (1981).
6. Bush, K., Jacoby, G. A., Medeiros, A. A.: A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39, 1211–1233 (1995).
7. Carter, J. S., Bowden, F. J., Bastian, I., Myers, G. M., Sriprakash, K. S., Kemp, D. J.: Phylogenetic evidence for reclassification of *Calymmatobacterium granulomatis* as *Klebsiella granulomatis* comb. nov. Int. J. Syst. Bacteriol. 49, 1695–1700 (1999).
8. Cavalcante, V., Döbereiner, J.: A new acid-tolerant nitrogen-fixing bacterium associated with the sugarcane. Plant Soil 108, 23–31 (1998).
9. Chelius, M. K., Triplett, E. W.: Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. Appl. Environ. Microbiol. 66, 783–787 (2000).
10. Combe, M.-L., Pons, J.-L., Sesboue, R., Martin, J. P.: Electrophoretic transfer from polyacrylamide gel to nitrocellulose sheets, a new method to characterize multilocus enzyme genotypes of *Klebsiella* strains. Appl. Environ. Microbiol. 60, 26–30 (1994).
11. Dong, Y., Chelius, M. K., Brisse, S., Kozyrovska, N., Kovtunovych, G., Podschun, R., Triplett, E. W.: Comparisons between two *Klebsiella*: The plant endophyte *K. pneumoniae* 342 and a clinical isolate *K. pneumoniae* MGH78578. Symbiosis 35, 247–259 (2003).
12. Drancourt, M., Bollet, C., Carta, A., Rousselier, P.: Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. Int. J. Syst. Evol. Microbiol. 51, 925–932 (2001).
13. Ferragut, C., Kersters, K., De Ley, J.: Protein electrophoretic and DNA homology analysis of *Klebsiella* strains. Syst. Appl. Microbiol. 11, 121–127 (1989).
14. Fournier, B., Roy, P. H., Lagrange, P. H., Philippon, A.: Chromosomal beta-lactamase genes of *Klebsiella oxytoca* are divided into two main groups, blaOXY-1 and blaOXY-2. Antimicrob. Agents Chemother. 40, 454–459 (1996).
15. Fuentes-Ramírez, L. E., Bustillos-Cristales, R., Tapia-Hernández, A., Jiménez-Salgado, T., Wang, E. T., Martínez-Romero, E., Caballero-Mellado, J.: Novel nitrogen-fixing acetic acid bacteria *Gluconacetobacter johannae* sp. nov. *and Gluconacetobacter azotocaptans* sp., nov., associated with coffee plants. Int. J. Syst. Evol. Microbiol. 51, 1305–1314 (2001).
16. Gordon, D. M., Lee, J.: The genetic structure of enteric bacteria from Australian mammals. Microbiol. 145, 2673–2682 (1999).
17. Granier, S. A., Plaisance, L., Leflon-Guibout, V., Lagier, E., Morand, S., Goldstein, F. W., Nicolas-Chanoine, M. H.: Recognition of two genetic groups in *Klebsiella oxytoca* taxon on the basis of the chromosomal β-lactamase and housekeeping gene sequences as well as ERIC-1R PCR typing. Int. J. Syst. Evol. Microbiol. (in press) (2002).
18. Grimont, F., Grimont, P. A. D., Richard, C.: The genus *Klebsiella*. In: *The Prokaryotes* (Balow A, Trüper HG, Dworkin M, Harder W, Schleifer K-H eds.) 1st. ed., New York, Springer Verlag 1992.
19. Grimont, F., Grimont, P. A. D., Richard, C.: The genus *Klebsiella*. In: *The Prokaryotes*, (Dworkin et al. eds.) 2nd. ed., New York, Springer Verlag 2000.
20. Haahtela, K., Korhonen, T. K.: In vitro adhesion of N2-fixing enteric bacteria to roots of grasses and cereals. Appl. Environ. Microbiol. 49, 1186–1190 (1985).
21. Hashimoto, J. G., Stevenson, B. S., Schmidt, T. M.: Rates and consequences of recombinaton between rRNA operons. J. Bacteriol. 185, 966–972 (2003).
22. Hedegaard J., Steffensen S. A., Norskov-Lauritsen N., Mortensen K. K., Sperling-Petersen H. U.: Identification of Enterobacteriaceae by partial sequencing of the gene encoding translation initiation factor 2. Int. J. Syst. Bacteriol. 4, 1531–1538 (1999).

23. Howard, D. J., Bush, G. L., Breznak, J. A.: The evolutionary significance of bacteria associated with *Rhagoletis*. Evolution 39, 405–417 (1985).
24. Hynes, M. F., McGregor, N. F.: Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol. Microbiol. 4, 567–574 (1990).
25. Jain, K., Radsak, K., Mannheim, W.: Differentiation of the *Oxytocum* group from *Klebsiella* by deoxyribonucleic acid-deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. 24, 402–407 (1974).
26. Kanki, M., Yoda, T., Tsukamoto, T., Shibata, T.: *Klebsiella pneumoniae* produces no histamine: *Raoultella planticola* and *Raoultella ornithynolytica* strains are histamine producers. Appl. Environ. Microbiol. 68, 3462–3466 (2002).
27. Knittel, M. D., Seidler, R. J., Eby, C., Cabe, L. M.: Colonization of the botanical environment by *Klebsiella* isolates of pathogenic origin. Appl. Environ. Microbiol. 34, 557–563 (1977).
28. Kovtunovych, G., Lar, O., Kamalova, S., Kordyum, V., Kleiner, D., Kozyrovska, N.: Correlation between pectate lyase activity and ability of diazotrophic *Klebsiella oxytoca* VN 13 to penetrate into plant tissues. Plant Soil 215, 1–6 (1999).
29. Ladha, J. K., Barraquio, W. L., Watanabe, I.: Isolation and identification of nitrogen-fixing *Enterobacter clocae* and *Klebsiella planticola* associated with rice plants. Can. J. Microbiol. 29, 1301–1308 (1983).
30. Lai, Y. -C., Peng, H. -L., Chang H. -Y.: Identification of genes induced in vivo during *Klebsiella pneumoniae* CG43 infection. Infection Immunity 69, 7140–7145 (2001).
31. Martínez, L., Caballero, J., Orozco, J., Martínez-Romero, E.: Diazotrophic bacteria associated with banana (*Musa* spp.). Plant Soil 257, 35–47 (2003).
32. Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., Pardo, M. A.: *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41, 417–426 (1991).
33. Matsen, J. M., Spindler, J. A., Blosser, R. O.: Characterization of *Klebsiella* isolates from natural receiving waters and comparison with human isolates. Appl. Microbiol. 28, 672–678 (1974).
34. McNeil, M., Darvill, A. G., Fry, S. C., Albersheim, P.: Structure and function of the primary cell walls of plants. Annu. Rev. Biochem. 53, 625–663 (1984).
35. Miller, J. H.: Experiments in molecular genetics, p. 431–435. Cold Spring Harbor, N. Y, Cold Spring Harbor Laboratory, 1972.
36. Mollet, C., Drancourt, M., Didier, R.: *rpoB* sequence analysis as a novel basis for bacterial identification. Mol. Microbiol. 26, 1005–1011 (1997).
37. Musser, J. M., Bemis, D. A., Ishikawa. H., Selander, R. K.: Clonal diversity and host distribution in *Bordetella bronchiseptica*. J. Bacteriol. 169, 2793–2803 (1987).
38. Noller, A. C., McEllistrem, M. C., Stine, O. C., Morris Jr., J. G., Boxrud, D. J., Dixon, B., Harrison, L. H.: Multilocus Sequence Typing reveals a lack of diversity among *Escherichia coli* O157:H7. J. Clin. Microbiol. 41, 675–679 (2003).
39. Palus, J. A., Borneman, J., Ludden, P. W., Triplett, E. W.: A diazotrophic bacterial endophyte isolated from stems of *Zea mays* L., and *Zea luxurians* Itlis and Doebley. Plant Soil 186, 135–142 (1996).
40. Podschun, R., Pietsch, S., Höller, C., Ullman, U.: Incidence of *Klebsiella* species in surface waters and their expression of virulence factors. Appl. Environ. Microbiol. 67, 3325–3327 (2001).
41. Podschun, R., Ullmann, U.: *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 11, 589–603 (1998).
42. Poly, F., Monrozier, L. J., Bally, R.: Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res. Microbiol. 152, 95–103 (2001).
43. Rivera, M. C., Jain, R., Moore, J. E., Lake, J. A.: Genomic evidence for two functionally distinct gene classes. Proc. Natl. Acad. Sci. U. S. A. 95, 6239–6244 (1998).
44. Rogel, M. A., Hernández-Lucas, I., Kuykendall, L. D., Balkwill, D. L., Martínez-Romero, E.: Nitrogen-fixing nodules with *Ensifer adhaerens* harboring *Rhizobium tropici* symbiotic plasmids. Appl. Environ. Microbiol. 67, 3264–3268 (2001).
45. Selander, R. K., McKinney. R. M, Whittam. T. S., Bibb, W. F., Brenner, D. H., Nolte, F. S., Pattison, P. E.: Genetic structure of populations of *Legionella pneumophila*. J. Bacteriol. 163, 1021–1037 (1985).
46. Silva, J., Aguilar, C., Becerra, Z., Lopez-Antunano, F., García, R.: Extended-spectrum β-lactamases in clinical isolates of enterobacteria in Mexico. Microb. Drug Resist. 5, 189–193 (1999).
47. Singh, M., Kleeberger, A., Klingmüller, W.: Location of nitrogen fixation (*nif*) genes on indigenous plasmids of *Enterobacter agglomerans*. Mol. Gen. Genet. 190, 373–378 (1983).
48. Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kampfer, P., Maiden, M. C., Nesme, X., Rossello-Mora, R., Swings, J., Trüper, H. G., Vauterin, L., Ward, A. C., Whitman, W. B.: Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52, 1043–1047 (2002).
49. Stackebrandt, E., Goebel, B. M.: Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44, 846–849 (1994).
50. Toledo, I., Lloret. L., Martínez-Romero, E.: *Sinorhizobium americanum* sp. nov., a new *Sinorhizobium* species nodulating native *Acacia* spp. in Mexico. Syst. Appl. Microbiol. 26, 54–64 (2003).
51. Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., Swings, J.: Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60, 407–438 (1996).
52. Vincent, J. M.: A Manual for the Practical Study of Root-nodule Bacteria. IBP Handbook No. 15, pp. 164. Oxford Blackwell Scientific Publications (1970).
53. Wang, E. T., Rogel, M. A., García-de los Santos, A., Martínez-Romero, J., Cevallos, M. A., Martínez-Romero, E.: *Rhizobium etli* bv. *mimosae*, a novel biovar isolated from *Mimosa affinis*. Int. J. Syst. Bacteriol. 49, 1479–1491 (1999).
54. Wang, E. T., van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X., Martínez-Romero, E.: *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. Int. J. Syst. Bacteriol. 48, 687–699 (1998).
55. Wang, E. T., van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X., Martínez-Romero, E.: Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. Int. J. Syst. Bacteriol. 49, 51–65 (1999).
56. Wong, S. H., Cullimore, D. R., Bruce, D. L.: Selective medium for the isolation and enumeration of *Klebsiella* spp. Appl. Environ. Microbiol. 49, 1022–1024 (1985).

Corresponding author:
Esperanza Martínez-Romero, Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Ap. P. 565-A., Cuernavaca, Morelos, México
Tel.: ++52-777-313-16-97; Fax: ++52-777-317-55-81;
e-mail: emartine@cifn.unam.mx