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Acetobacter diazotrophicus sp. nov., a Nitrogen-Fixing Acetic Acid Bacterium Associated with Sugarcane

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Results of deoxyribonucleic acid (DNA)-ribosomal ribonucleic acid and DNA-DNA hybridizations, together with a phenotypic and chemotaxonomic analysis, revealed that nitrogen-fixing bacteria isolated from roots and stems of sugarcane belong to a new species in the genus *Acetobacter*, for which the name *Acetobacter diazotrophicus* sp. nov. is proposed. Strain LMG 7603 (= Döbereiner PAl 5 = ATCC 49037) is the type strain.

New microaerobic, gram-negative, N_2-fixing bacteria were isolated from roots and stems of sugarcane in Brazil (1). Because of their ability to grow at low pH values and their ability to form acetic acid from ethanol, these organisms could belong to the acetic acid bacteria (the genera *Acetobacter* and *Gluconobacter*) (3, 4) or to the genus *Frateuria* (14). However, Cavalcante and Döbereiner (1) believed that the differences between these genera and the N_2-fixing sugarcane isolates were sufficiently great to propose the name "*Saccharobacter nitrocaptans*" (not validly published). The original description (1) was based on more than 20 strains isolated in four different regions of Brazil.

In this paper we present genomic, phenotypic, and chemotaxonomic evidence that these isolates constitute a new species in the genus *Acetobacter*, for which we propose the name *Acetobacter diazotrophicus*.

Details of the strains and their sources are given in Table 1. Cells for genomic studies, protein electrophoresis, and determination of ubiquinones were grown on GYC medium (5% glucose, 1% yeast extract, 3% $CaCO_3$, 2.5% agar). The methods which we used have been described previously (2, 4, 6–12). Differentiating phenotypic tests were simultaneously carried out on all *A. diazotrophicus* strains listed in Table 1; the type strains of all *Acetobacter* species were included as controls.

The protein electrophoretic fingerprints of the seven *A. diazotrophicus* strains are very similar, indicating that these sugarcane isolates are highly related to each other (Fig. 1), yet the protein patterns of strains PR 14 and PR 2 are slightly different from those of the other sugarcane isolates. The levels of deoxyribonucleic acid (DNA) binding (as determined by the initial renaturation rate method [2]) among three representative *A. diazotrophicus* strains (strains PA 5^T [T = type strain], PPe 4, and PR 2) were more than 84%, indicating that these strains form a single DNA homology group.

DNA-ribosomal ribonucleic acid (rRNA) hybridizations revealed the generic status of the sugarcane isolates. A preliminary hybridization between DNA from strain PA 5^T and 23S [^{14}C]rRNA from *Xanthomonas campestris* NCPPB 528^T resulted in a $T_{m(e)}$ value (midpoint temperature of the thermal denaturation curve of the DNA-rRNA hybrid) of 55.6°C, demonstrating that the sugarcane isolates are not members of rRNA superfamily II, which corresponds to the gamma subclass of the *Proteobacteria* (13). This means that they are not related to the genus *Frateuria*, which is located on the *Xanthomonas* rRNA branch (5, 14). With 23S [^{14}C]rRNA from *Gluconobacter oxydans* NCIB 9013^T we found mean $T_{m(e)}$ values of 76.1 and 77.0°C for strains PPe 4 and PAl 5^T, respectively. This shows that these strains belong to the family *Acetobacteraceae* (6) and more specifically to the *Acetobacter* rRNA cluster (with $T_{m(e)}$ values ranging from 74.5 to 78.3°C versus 23S [^{14}C]rRNA from *G. oxydans* NCIB 9013^T). The family *Acetobacteraceae* constitutes a separate rRNA branch in rRNA superfamily IV, which corresponds to the alpha subclass of the *Proteobacteria* (13). Also, the peritrichous flagellation of the sugarcane isolates and their ability to oxidize acetic acid and lactic acid to CO_2 (1) confirm that these organisms belong in the genus *Acetobacter* (4). The other phenotypic features shown in Table 2, together with the formation of acid from *n*-propanol and *n*-butanol and the absence of gelatin liquefaction, indole formation, and hydrolysis of lactose and starch, agree with the description of the genus *Acetobacter* (4). This conclusion is also supported by the results of an analysis of the cellular fatty acids of strains PA 5^T and PR 2. We found large amounts (>50%) of $C_{18:1}$ acid and small amounts of $C_{14:0}$ acid, which is typical of the family *Acetobacteraceae* and the genus *Acetobacter*, respectively (18).

Within the genus *Acetobacter* the following four species were described in *Bergey's Manual of Systematic Bacteriology*, vol. 1 (4): *Acetobacter aceti*, *Acetobacter liquefaciens*, *Acetobacter pasteurianus*, and *Acetobacter hansenii*. Since then, the following additional proposals have been published: the revival of *Acetobacter xylinum* (16, 17), the creation of a new subgenus *Gluconoacetobacter* within the genus *Acetobacter* to contain the acetobacters with ubiquinone Q10 (17), and the description of a new species, *Acetobacter methanolicus* (15). We found that none of these *Acetobacter* species was able to fix N_2 in semisolid medium, even when various concentrations of yeast extract (50 to 500 mg/liter of medium) were added as an N source for growth. Other phenotypic features differentiating the sugarcane isolates from the other *Acetobacter* species are given in Table 2; the sugarcane isolates have more phenotypic characteristics in common with *A. liquefaciens* than with the other species.

The protein electropherograms of the sugarcane isolates differed from those of all of the reference strains examined, although some similarities with the patterns of *A. liquefaciens* were found (Fig. 1). However, strains PA 5^T, PR 2,

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TABLE 1. Strains used

Strain[a]	Other designation(s)[a]	Source
A. diazotrophicus		
PR 2	LMG 8067, ATCC 49039	Sugarcane roots, Rio de Janeiro, Brazil
PR 14	LMG 8068	Sugarcane rhizosphere, Rio de Janeiro, Brazil
PR 4	LMG 8065	Sugarcane stems, Rio de Janeiro, Brazil
PPe 4	LMG 7971, ATCC 49038	Sugarcane stems, Pernambuco, Brazil
PAl 2	LMG 7602	Sugarcane roots, Alagoas, Brazil
PAl 5^T	LMG 7603^T, ATCC 49037^T	Sugarcane roots, Alagoas, Brazil
PAl 3	LMG 8066	Sugarcane roots, Alagoas, Brazil
A. aceti LMG $1261t1^T$	NCIB 8621^T	Beechwood shavings in vinegar generator
A. hansenii LMG 1527^T	NCIB 8746^T	Vinegar
A. liquefaciens		
LMG 1381^T	NCIB 9136^T	Dried fruit of *Diospyros kaki*
LMG 1728	Cho 222	Pink-diseased pineapple fruit
A. methanolicus LMG 1668^T	$MB58^T$	Septic methanol-yeast process
A. pasteurianus LMG $1262t2^T$	LMD 22.1^T	Beer
A. xylinum LMG 1515^T	NCIB 11664^T	Probably from juice of *Sorbus aucuparia* berries

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; LMD, Laboratorium voor Microbiologie, Technische Hogeschool, Delft, The Netherlands; LMG, Culture Collection Laboratorium voor Microbiologie, Ghent, Belgium; NCIB, National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland.

and PPe 4 displayed no significant degree (less than 25%) of DNA binding either with the type strain of *A. liquefaciens* or with any other type strain of an *Acetobacter* species. Genotypically, the sugarcane isolates constitute a distinct group within the genus *Acetobacter*. Because this group can also be differentiated phenotypically from all of the other *Acetobacter* species (Table 2), we propose for it a new species, *Acetobacter diazotrophicus*. The ubiquinone type is Q10; small amounts (less than 3%) of ubiquinone Q9 were also found. Therefore, like *A. methanolicus*, *A. diazotrophicus* can be assigned to the subgenus *Gluconoacetobacter*, if the subgenus subdivision described by Yamada and Kondo (17) is followed. The description below is based on the description of Cavalcante and Döbereiner (1) and the results of our work (Table 2) and does not repeat the phenotypic features from the description of the genus *Acetobacter* (4).

Description of *Acetobacter diazotrophicus* sp. nov. *Acetobacter diazotrophicus* (di.a.zo.tro'phi.cus.Gr. prefix *di*, two, double; N.L.n. *azotum*, nitrogen; Gr.adj. *trophikos*, nursing, tending or feeding; M.L. masc. adj. *diazotrophicus*, one that feeds on dinitrogen). Cells are straight rods with rounded ends, about 0.7 to 0.9 by ±2 μm. Long involution forms occur in unshaken rich liquid media. Motile by lateral or peritrichous flagella (Fig. 2). Produces brown water-soluble pigments on GYC medium. Dark brown colonies are formed on potato agar supplemented with 10% sucrose, and dark orange colonies are formed on a nitrogen-poor medium containing bromothymol blue (1). Forms 2-ketogluconic acid and 2,5-diketogluconic acid from glucose. These organisms are microaerobic dinitrogen fixers which grow on N_2 as a sole nitrogen source in semisolid media and after initial growth with starter nitrogen in liquid media; they grow well in liquid media with combined nitrogen sources. Nitrate is not reduced, and N_2 fixation occurs at high nitrate concentrations (10 mM). The optimum growth temperature is around 30°C, and the optimum pH is 5.5. Does not grow at pH 7.0, but does grow and fix N_2 at pH values below 3.0. H_2S is not formed from L-cysteine, except by some strains when they are grown in a medium containing sucrose instead of glucose. Grows well in mineral media containing NH_4^+. Growth is poor on organic acids, but acetate and lactate are oxidized to CO_2 and water. High concentrations (10%) of sucrose are the best carbon source for growth, but glucose, fructose, and galactose are also used. Growth and N_2 fixation occur with 30% glucose or 30% sucrose. Ethanol (1%), mannitol, and glycerol are also used as carbon sources. The major cellular fatty acid is $C_{18:1}$ acid. The ubiquinone type is Q10. Other features are listed in Table 2. The average guanine-plus-cytosine content of the DNA ranges from 61 to 63 mol% (as determined by the thermal

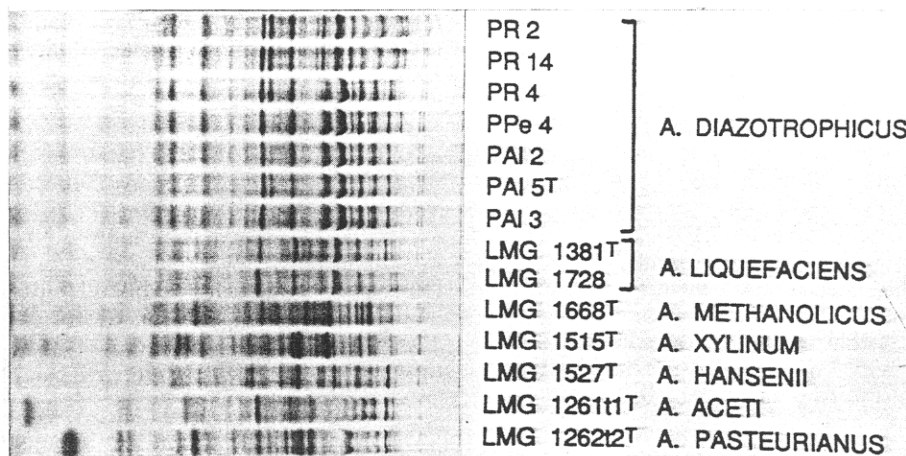

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic cellular protein patterns of seven *A. diazotrophicus* strains and the type strains of the other *Acetobacter* species.

TABLE 2. Differentiating characteristics for *A. diazotrophicus* and the other *Acetobacter* species[a]

Characteristics	*A. diazotrophicus*[b]	*A. aceti*[c]	*A. liquefaciens*[c]	*A. pasteurianus*[c]	*A. hansenii*[c]	*A. xylinum*[d]	*A. methanolicus*[e]
Formation of:							
Water-soluble brown pigments on GYC medium	+	−	+	−	−	−	−
γ-Pyrones from D-glucose	+	−	d	−	−	−	−
γ-Pyrones from D-fructose	+	−	+	−	−	−	−
5-Ketogluconic acid from D-glucose	−	+	d	−	d	+	−
2,5-Diketogluconic acid from D-glucose	+	−	+	−	−	−	−
Ketogenesis from glycerol	d	+	+	−	+	+	(+)
Growth factors required[f]	−	d	d	+	d	+	+
Growth on the following carbon sources:							
Ethanol	+	+	+	d	−	−	(+)
Methanol[g]	−	−	−	−	−	−	+
Dulcitol	−	−	−	−	d	−	−
Sodium acetate	+	+	d	d	−	−	(+)
Growth on the following L-amino acids in the presence of D-mannitol as a carbon source:							
L-Glycine, L-threonine, L-tryptophan	−	−	d	−	−	−	−
L-Asparagine	+	d	+	−	+	−	−
L-Glutamine	−	d	+	−	+	−	−
Growth in the presence of 10% ethanol	−	−	−	d	−	−	−
Formation of H_2S	−[h]	−	−	−	−	−	−
Growth in the presence of 30% D-glucose[i]	+	−	−	−	−	−	−
N_2 fixation and growth on dinitrogen[i]	+	−	−	−	−	ND	−
Ubiquinone type	Q10	Q9	Q10	Q9	ND	Q10	Q10
Guanine-plus-cytosine content of DNA (mol%)	61–63	56–60	62–65	53–63	58–63	55–63	62

[a] +, 90% or more of the strains are positive; (+), weakly positive reaction; d, 11 to 89% of the strains are positive; −, 90% or more of the strains are negative; ND, not determined.
[b] Data from reference 1 and this study.
[c] Data from reference 4, except for the characteristics indicated.
[d] Results for type strain LMG 1515 only. Data from F. Gosselé (Ph.D. thesis, State University of Ghent, Ghent, Belgium, 1982), except for the characteristics indicated.
[e] Data from reference 15, except for N_2 fixation, which was only determined for the type strain.
[f] Data from Gosselé (Ph.D. thesis), except the data for *A. diazotrophicus*.
[g] Our results for type strains only, except the data for *A. methanolicus* (15).
[h] Some strains were positive on sucrose media.
[i] Data for all of the *A. diazotrophicus* strains and the type strains of the other species (data from reference 1 and this study).

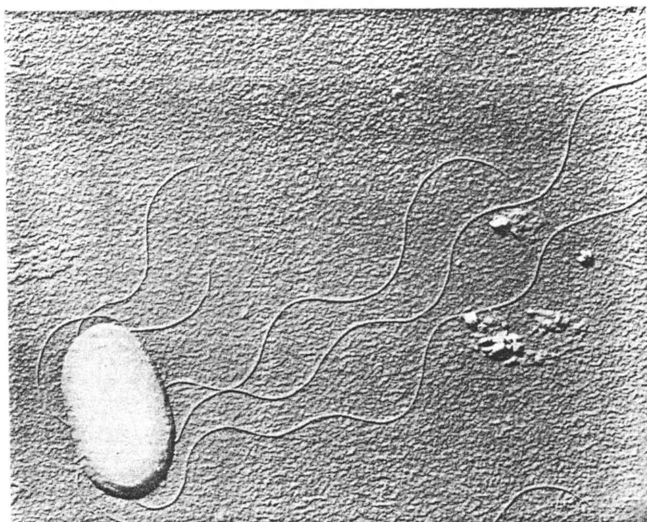

FIG. 2. Electron micrograph of a cell of *A. diazotrophicus* PA 5[T], showing the peritrichous flagellar arrangement. The cell dimensions are 0.7 to 0.9 by 2 μm.

denaturation method). The habitat is roots and stems of sugarcane. The type strain is strain LMG 7603 (= Döbereiner PA 5 = ATCC 49037).

Description of the type strain. *A. diazotrophicus* LMG 7603[T] has all of the characteristics given above for the species. Ketogenesis from glycerol is positive, and the guanine-plus-cytosine content of the DNA is 61 mol%. This strain was isolated by V. Cavalcante and J. Döbereiner in 1987 from sugarcane roots in Alagoas, Brazil.

Recently, we found that sugarcane isolates from Australia also belong in *A. diazotrophicus* according to their growth characteristics, protein gel electropherograms, and N_2-fixing ability.

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