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Further Evidence that the N_2-Fixing Endophytic Bacterium from the Intercellular Spaces of Sugarcane Stems Is *Acetobacter diazotrophicus*

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Nitrogen-fixing bacteria, isolated from the sugar solution in intercellular spaces of sugarcane stems, were compared with the type strain of *Acetobacter diazotrophicus* (PAL-5) and found to be congruent with it in all characters studied. These characters were 37 morphological and biochemical tests, cellular fatty acid composition, and nitrogenase activity. The nitrogenase activity was measured by acetylene reduction and H_2 evolution and found to be unusual in that the H_2 evolution was suppressed much less than expected by high concentrations of acetylene.

Nitrogen-fixing, acid-producing, and acid-tolerant gram-negative bacteria have been isolated from the fluid in intercellular spaces of sugarcane stems (9). These isolates grow best on a sucrose-rich medium and can continue to grow in up to 30% sucrose. Previously, we had found, by use of a small number of biochemical tests, that these bacteria were culturally, metabolically, and phenotypically similar to the N_2-fixing species *Acetobacter diazotrophicus*, which was first described in 1989 (9, 13). In the present report, we provide a more complete description of the phenotypic and cultural characteristics of this bacterium by use of a highly comprehensive panel of conventional biochemical tests and determination of cellular fatty acid (CFA) composition by a modern automated system.

The production of H_2 by the isolates has been compared with that of the type strain because this gas is an obligatory product of nitrogenase activity, resulting in N_2 fixation. The level of H_2 production can be used to monitor the activity of the N_2-fixing process (16, 22). This H_2 production is suppressed to different degrees in different N_2-fixing bacteria by the presence of acetylene (8). The extent of the suppression has been used to further characterize the isolates from the sugarcane stems.

MATERIALS AND METHODS

Plants. Two varieties of sugarcane (*Saccharum officinarum* L.), cv. Media Luna and JA60-5, which had been cultivated in the field in Havana, Cuba, or were first-, second-, or third-generation subcultures of Cuban stocks propagated vegetatively in a greenhouse in Ottawa, Ontario, Canada, were used (9).

Bacterial strains. Isolates were obtained by inoculating the fluid removed by aseptic centrifugation from intercellular spaces of the sugarcane stems into semisolid LGI medium (9). Only those eight isolates obtained from different plants which were capable of reducing acetylene were compared. The type strain of *A. diazotrophicus*, with which all isolates were compared, was PAL-5 (ATCC-49037 ex Döbereiner, from the culture collection of the Departamento de Microbiologia, Universidad de La Habana). The strains designated MC-1, -2, -3, -4, and -7 came from cv. Media Luna (field material from Havana, Cuba), and those designated JO-1, -2, and -4 came from cv. JA60-5 (Carleton University greenhouse). All bacteria were stored in 10% glycerol in a liquid N_2 storage apparatus. Bacteria newly subcultured from these frozen cultures on slopes of modified LGI medium (6), a nitrogen-free, sucrose-rich (100 g/liter) medium supplemented with sugarcane juice (5 ml/liter), were used throughout the study.

Conventional morphological criteria and biochemical tests. All of the tests and morphological observations made on the isolates are listed in Table 1. These include those used in an earlier study (9). All tests were done at 30°C in air.

CFA analysis. Cells for fatty acid analysis were grown on LGI medium at 30°C for 6 days and saponified, methylated, and analyzed by gas-liquid chromatography with the Microbial Identification System and Library Generation System software (MIDI, Newark, Del.) as described previously (2).

At the Laboratory Center for Disease Control, tentative peak identifications were made on the basis of the use of a reference standard of fatty acids obtained from MIDI. Chromatographic profiles were created and updated for storage in an in-house library for aerobes and facultative anaerobes called LCDC1 (2), whose method, LCDCAER2, was rooted to the commercial method AEROBE (MIDI), version 3.8, and validated with Library Generation System software, including principal-component analyses. CFA profiles of all strains were tested against the commercial aerobe libraries TSBA and CLIN version 3.8 (MIDI), which do not contain *A. diazotrophicus*.

Measurements of hydrogen production. Cultures were grown on solid medium in 36-ml glass tubes containing 10 ml of LGI medium at 30°C for 5 days before the test. This temperature was maintained throughout the test by placing the tubes in a water bath. The H_2 evolution was measured as follows. The tubes were sealed with gas-tight serum stoppers. Gas samples (1 ml) were withdrawn with syringes at about 10-min intervals and analyzed. After making measurements for 1 to 2 h to obtain the rate of H_2 production at 0% acetylene, the tubes were flushed with air for 5 min and resealed with serum stoppers. Acetylene was then added to the tubes by removing measured volumes of air and adding known volumes of pure acetylene by syringe to create partial pressures of 2.5, 10, 20, and 30 kPa. Gas samples were taken again at about 10-min intervals and analyzed for both H_2 and ethylene. Values from two replicate tubes each for all of the isolates and the type strain were compared.

Detached nodulated roots of soybean were tested like the gas samples for H_2 and ethylene production under the different partial pressures of acetylene as a further control.

To test whether the H_2 was produced by nitrogenase rather than by some other enzyme system, the rates of H_2 evolution in oxygen-nitrogen mixtures by the type strain and one of the isolates (MC-1) were compared with the rates in oxygen-argon mixtures. In the absence of its substrate (N_2), H_2 evolution from the nitrogenase should increase dramatically up to a theoretical maximum of fourfold (10).

All analyses were done by immediately injecting each gas sample into a portable gas chromatograph which was equipped with a semiconductor sensor (Taguchi gas sensor; Figaro Engineering Co., Toyonaka City, Japan) with Porapak N (0.63 m by 0.2 mm, internal diameter) at room temperature (15). Chromatograms with retention times and peak heights and areas were recorded with a computing integrator (LCI-100; Perkin-Elmer). The gases were identified tentatively by comparing retention times with those of standard gases. The sample gas was also spiked by assaying the mixture of standard gas of known concentration with sampled gas. Chromatograms were compared with those of standard gas and sample gas alone to ensure the identification of each peak present.

There was no possible confusion between the H_2 peak and a carbon dioxide peak, since the sensor was very insensitive to carbon dioxide.

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TABLE 1. Standard morphological and biochemical characteristics of *A. diazotrophicus* type strain and isolates[a]

Test	Type strain PAL-5	Isolates							
		MC-1	JO-1	MC-2	JO-2	MC-3	MC-4	JO-4	MC-7
Gram stain[b]	−	−	−	−	−	−	−	−	−
Endospore[b]	−	−	−	−	−	−	−	−	−
Motility[b]	+	+	+	+	+	+	+	+	+
Flagellae peritrichous[c]	+	+	+	+	+	+	+	+	+
Nitrate reduction[d]	−	−	−	−	−	−	−	−	−
Catalase[b,c]	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis[b]	−	−	−	−	−	−	−	−	−
Starch hydrolysis[b]	−	−	−	−	−	−	−	−	−
Oxidase[b]	−	−	−	−	−	−	−	−	−
Overoxidation of ethanol[e]	+	+	+	+	+	+	+	+	+
Overoxidation of 1% glucose[b]	+	+	+	+	+	+	+	+	+
Brown pigment on GYC agar[e]	+	+	+	+	+	+	+	+	+
Brown colonies on potato agar[f]	+	+	+	+	+	+	+	+	+
Growth with 30% glucose	+	+	+	+	+	+	+	+	+
Growth with 30% sucrose[c]	+	+	+	+	+	+	+	+	+
Oxidation of glycerol[b]	+	+	+	+	+	+	+	+	+
Formation of H_2S[b]	−	−	−	−	−	−	−	−	−
Growth on LGI-P plates[c,f]	+	+	+	+	+	+	+	+	+
Methyl red[b]	−	−	−	−	−	−	−	−	−
Growth on carbon sources[e]:									
D-Glucose	+	+	+	+	+	+	+	+	+
D-Arabinose	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+
i-Inositol	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
Acid production from[e]:									
D-Glucose	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+
i-Inositol	−	−	−	−	−	−	−	−	−
D-Mannitol	−	−	−	−	−	−	−	−	−
D-Arabinose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
Growth on amino acids with D-mannitol[e]									
L-Glycine	−	−	−	−	−	−	−	−	−
D,L-Tryptophan	−	−	−	−	−	−	−	−	−
L-Asparagine	±	±	±	±	±	±	±	±	±
L-Threonine	−	−	−	−	−	−	−	−	−
L-Glutamine	±	±	±	±	±	±	±	±	±

[a] +, positive; −, negative; ±, slight growth.
[b] Methods from Gerhardt et al. (12).
[c] From Table II of Dong et al. (9).
[d] Methods from Pelczar and Chen (20).
[e] Methods from De Ley et al. (7).
[f] Methods from Cavalcante and Döbereiner (6).

RESULTS

All of the isolates and the type strain were identical for the morphological and biochemical characteristics examined. These results are summarized in Table 1.

The CFA composition, expressed as percentages for the type strain and the isolates, is presented in Table 2. All strains were found to have no match with all entries contained in library CLIN. However, all strains had the genus *Acetobacter* as the only choice when tested against the TSBA library, with similarity index values ranging from 0.185 to 0.481. The *Acetobacter* species picked up by the system from the TSBA library were *A. xylinum*, *A. pasteurianus*, and *A. liquefaciens*. The principal component 1 value for all data when averaged into a library entry with the Library Generation System software was 1.25.

In a previous paper (9), nitrogenase activity was estimated by the reduction of acetylene to produce ethylene. We now show that this activity can also be assayed by the production of H_2 by cultures growing in air. This H_2 production was detected from the type strain and all isolates and continued at a steady rate for several hours. In an individual culture tube (10-ml medium slope), the production of H_2 is about 60 nmol/h. In the presence of acetylene (e.g., when using this test as a nitrogenase assay), both H_2 and ethylene are produced, but the H_2 is produced at a reduced rate. The reduction increases as the acetylene concentration rises, but even at 30 kPa, the rate is substantial (Table 3). The rates of H_2 production in the presence of acetylene remained steady over at least 2 h. This behavior is in strong contrast to that of soybean nodules, whose H_2 production is strongly suppressed by acetylene. The contrast is shown in Table 3, where the gas production rates by individual cultures and nodule preparations have been normalized to 100% in air. The results for the type strain and the three isolates tested were not significantly different and have been pooled to give the means in Table 3. As the acetylene concen-

TABLE 2. CFA composition analysis of sugarcane isolates and *A. diazotrophicus* ATCC 49037[a]

Strain	% CFA[b]										
	14:0	2-OH-14:0	3-OH-14:0	16:0	2-OH-16:0	3-OH-16:0	18:2	18:1,ω7c	18:0	19:0,cycω8c	3-OH-18:0
Type strain ATCC 49037 (PAL-5)	5	3	2	14	6	4	1	55	2	5	2
Isolates											
JO-1	5	3	2	13	6	3	1	57	3	6	2
JO-2	6	4	2	13	7	3	—	57	2	5	2
JO-4	5	3	2	14	6	3	1	56	2	6	2
MC-1	6	3	1	13	6	3	2	57	3	5	2
MC-2	6	4	2	13	7	3	—	58	2	5	2
MC-3	5	3	2	14	6	3	—	57	2	5	2
MC-4	5	3	2	13	6	3	2	56	2	5	2
MC-7	5	4	1	13	7	3	2	55	2	6	2
Mean	5.4	3.4	1.6	13.1	6.4	3.4	1.03	56.4	2.3	5.3	1.8
SD	0.2	0.3	0.1	0.5	0.2	0.2	0.06	0.9	0.3	0.5	0.2

[a] Trace to small volumes (1 to 9%) of peaks identified with equivalent chain lengths of 18.588, 18.717, 19.710, and 20.188 were observed for all or most sugarcane isolates and are observed among CFA compositions for a number of genera of gram-negative bacilli (1). The CFA which elutes at an equivalent chain length of 20.188 has been characterized previously by Wallace et al. (24) and is identifiable as 2OH-19:0cyc$_{11-12}$.

[b] For fatty acid designations, the number to the left of the colon is the number of carbon atoms, and the number to the right is the number of double bonds. Other symbols: ω, double bond position from the hydrocarbon end of the chain; c, *cis* isomer; OH, hydroxyl group at second or third carbon; cyc, cyclopropane form. The MIDI peak naming table (v3.8) more commonly refers to 3-OH-14:0 as summed feature 3, 18:2 as part of summed feature 6, and 18:1,ω7c as part of summed feature 7. Values shown are percentages of total fatty acids for the nine individual isolates, rounded off to the nearest percentages. The last two lines give the means and standard deviations for the nine isolates. —, 0 to 0.29%. Bernard et al. (3) reported that in the peak-naming table of AEROBE version 3.8, MIDI acknowledged that they had in error used 20:3 instead of 3-OH-18:0 for the CFA with an equivalent chain length of 19.547.

tration increases, H_2 production decreases and ethylene production increases, but the effect on the bacteria is much less than that on the nodules. At the concentration of acetylene commonly used in nitrogenase assays (i.e., 10 kPa), the H_2 production by the bacteria was reduced to 65%, but that by the nodules was reduced to only 11%. At a partial pressure of acetylene of 30 kPa, the H_2 production by the bacteria was still 15%, but that by the nodules was only 0.1%.

The results of tests on whether the H_2 produced by the *Acetobacter* spp. was indeed the result of nitrogenase activity and not by some other metabolic pathway are shown in Table 4. The isolate tested and the type strain gave similar results, with H_2 production rising when N_2 is replaced by argon. When the oxygen concentration in the argon mixture is 2%, the stimulation of H_2 production rises to threefold. Although this is less than the theoretical value of fourfold, it is good evidence that the H_2 is in fact produced by nitrogenase and not some other enzyme.

DISCUSSION

We found that all sugarcane isolates, whether recovered from field-grown plants in Cuba or from greenhouse-propagated stocks in Ottawa, Ontario, Canada, had phenotypic and morphological characteristics identical to each other and to those of the type strain of *A. diazotrophicus* for all properties listed in Table 1. All strains studied were consistent with each other both qualitatively and quantitatively for CFAs detected with the Microbial Identification System software (Table 2). All bacteria were found to exhibit low similarity index values solely towards species in the genus *Acetobacter* (*A. xylinum*, *A. pasteurianus*, and *A. liquefaciens*) when compared with the commercial library TSBA. As discussed in a review by Welch (25), only bacteria which are considered to be very closely related genetically will demonstrate highly similar CFA compositions; this supports the assertion here that the sugarcane isolates and the type strain of the species *A. diazotrophicus* have a relationship with other members of the *Acetobacter* genus or closely related genera. It was observed that the principal component 1 value for the entry created for all bacteria studied was 1.25. It has been suggested (19, 25) that such a low variance for CFA composition data among strains considered to be of the same species is suggestive of genetic homogeneity, which must be corroborated by additional molecular typing techniques.

The stability and persistence of this endophytic species under these widely different cultural conditions are in keeping with the findings of Caballero-Mellado and Martinez-Romero

TABLE 3. Relative rates of hydrogen and ethylene production by *A. diazotrophicus* and soybean nodules at different acetylene partial pressures in air

Culture or prepn	Gas produced	% Relative production rate (mean ± SD)[a]				
		0[b]	2.5	10	20	30
Acetobacter culture	H_2	100	84 ± 4	65 ± 5	26 ± 5	15 ± 3
	C_2H_4	0	62 ± 13	145 ± 27	159 ± 40	179 ± 35
Nodule prepn	H_2	100	16 ± 5	11 ± 4	1.7 ± 1.1	0.08 ± 0.07
	C_2H_4	0	108 ± 16	262 ± 34	254 ± 47	265 ± 38

[a] The results are normalized means of three tests, each one including the type strain and isolates.

[b] Acetylene partial pressure in kilopascals.

TABLE 4. Effect on hydrogen production by *A. diazotrophicus*[a] of replacing the nitrogenase substrate, nitrogen, with argon at high and low oxygen concentrations

Oxygen concn (%)	H_2 production[b] in:	
	Nitrogen-oxygen	Argon-oxygen[c]
20	100	159 ± 1.6
2	100	300 ± 11

[a] Isolate MC-1 and the type strain.
[b] Hydrogen production is expressed as the percentage relative to 100% in the nitrogen-oxygen mixture, as the mean of four replicates, each containing two tubes of the type strain and two of the isolate.
[c] Values are means ± standard deviations.

(5) that there is little genetic diversity among isolates of this bacterium from macerates of different varieties of sugarcane plants growing in diverse regions of Mexico and Brazil.

The finding that *A. diazotrophicus* has unusual properties among known N_2-fixing bacteria with respect to its continued production of H_2 in the presence of acetylene is new. Acetylene inhibition of H_2 production is well known for *Rhizobium* spp. and results from saturation of nitrogenase activity which occurs at a partial pressure of acetylene of about 10 kPa (14). The remote possibility that the H_2 produced by *A. diazotrophicus* originates by another enzyme system cannot be tested simply. The nitrogenase of this bacterium is not inhibited much by the presence of combined nitrogen (11, 17), so that the normal expedient of testing for the H_2 evolution in the presence of fixed nitrogen cannot be adopted. However, the markedly increased production of H_2 in the absence of the N_2 substrate of this enzyme suggests strongly that the H_2 does originate from protons liberated by the nitrogenase system and not another enzyme system. *A. diazotrophicus* is not unique in continuing to reduce N_2 (and liberate H_2) in the presence of much higher acetylene concentrations. Similar results have been reported by Spiff and Odu (23) and MacRae (18) for some *Beijerinckia* strains and by Dilworth (8) for *Azotobacter* strains.

In the *Azotobacter* strains, this continuation of H_2 production in the presence of high acetylene concentrations has been shown to be due to an alternative nitrogenase, synthesized only under extreme molybdenum starvation (4, 21). However, a similar nitrogenase is not acting in the continuing production of H_2 by *A. diazotrophicus* in the presence of high acetylene concentrations because normal levels of molybdenum (2 ppm Na_2MoO_4) were always present in the medium.

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