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A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane

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Abstract

During surveys of bacteria possibly responsible for N_2 fixation in sugarcane, root and stem samples were collected in four sugarcane-growing regions in Brazil. A new microaerobic N_2-fixing bacterium was isolated from most samples of washed roots and stems from all regions. Isolation procedures were based on semisolid diluted sugarcane juice medium followed by replication to N-free 10% sugar medium acidified with acetic acid to pH 4.5. The new bacterium is an aerobic rod, motile by 1 to 3 lateral flagella, fixes N_2 in semisolid media under air but not in liquid media except when a starter dose of N is added. It has no nitrate reductase and N_2 fixation proceeds in the presence of 10 m*M* NO_3^-. Best growth occurs with high sucrose concentrations (10%). Growth occurs up to 30% sucrose but not at 35%. Acid is formed reaching a final pH of below 3.0. Growth and N_2 fixation proceed at this acidity. Ethanol is used for growth and is "overoxidised" (oxidized to CO_2 and H_2O). Acetic and lactic acids are also oxidized to CO_2 and H_2O. Acids produced from glucose are consumed with precipitation of $CaCO_3$. Dark brown colonies are formed on potato agar with 10% sugar and dark orange colonies on N poor agar (20 mg yeast extract per l) containing bromothymol blue. In view of the distinct characteristics which do not allow identification within either Frateuria, Gluconobacter, Acetobacter or any known N_2-fixing bacterium a new genus and species are proposed and named *Saccharobacter nitrocaptans*.

Introduction

Sugarcane in Brazil is grown, not only for sugar, but in recent years it has become the basis for an ambitious fuel-alcohol project with more than 10 billion litres annually available for the replacement of fossil fuels. The success of such a project is invariably dependent on production systems with a positive energy balance as have been in use for decades in Brazil. Low input agriculture is typical for the traditionl North-East sugarcane areas but increased yields with continuing positive energy balances must rely on increased efficiency and or partial replacement of N fertilizers. Recently very promising results were obtained by N balance and ^{15}N isotope dilution studies (Lima *et al.*, 1987) indicating that substantial amounts of N_2 can be fixed by certain sugarcane cultivars. Nevertheless no data are available yet as to the responsible N_2-fixing bacteria.

The first observations on selective stimulation of N_2-fixing bacteria in sugarcane were reported many years ago in Brazil (Dobereiner and Alvahydo, 1959; Dobereiner, 1961). While 95% of more than 100 sugarcane soil samples contained Beijerinckia, only 60% of soil samples collected under other crops contained the bacterium. Also in the sugarcane rhizosphere and on the root surface there were 20 to 50 times more Beijerinckia and 2 to 5 times less other microorganisms than in control soil (Dobereiner 1961). Attempts to assess nitrogen fixation by the C_2H_2 reduction method indicated that most nitrogenase activity occurred in the rhizosphere soil but not in roots (Dobereiner *et al.*, 1972; Ruschel, 1979). Various methods have been used to identify other responsible diazotrophs

(Rennie, 1981; Rennie *et al.*, 1982) which seemed to yield almost exclusively Enterobacteriaceae isolated from the root surface. The two sugar-using *Azospirillum* spp, *A. lipoferum* and *A. amazonense* (unpublished observations from J Dobereiner) and a new *Bacillus* sp., *B. azotofixans* (Seldin *et al.*, 1984) were isolated from sugarcane roots from various sites in Brazil and from Hawaii, and unidentified diazotrophs were found to be present within sugarcane cuttings which multiplied and became active only after the emergence of roots (Patriquin *et al.*, 1980).

Here we report the occurrence of an entirely different new N_2-fixing bacterium in sugarcane roots and stems collected in various regions in Brazil.

Materials and methods

Sugarcane roots and stems were collected in various sugarcane regions from several cultivars. More details of the origin of samples are summarized in Table 1. Roots and stems were washed in tap water, macerated in a blender and serial dilutions prepared in sugar solution (5% cane sugar in H_2O) and inoculated into various enrichment media:

A. Semisolid LGI medium consisted of (quantities per litre): K_2HPO_4, 0.2 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; bromothymol blue 0.5% solution in 0.2*N* KOH, 5 ml, agar, 1.8 g; crystallized cane sugar, 100 g; final pH 6.0.

B. Acetic LGI medium was acidified with acetic acid to pH 4.5 (Micales *et al.*, 1985) and the agar concentration increased to 2.2 g per l.

C. Diluted cane juice medium contained 250 ml of medium A and 250 ml of sugarcane juice complemented with H_2O to 1 l.

D. LGI medium plates used for isolation contained the same ingredients as medium A but 0.02 g of Difco yeast extract and 15 g of agar were added.

E. Potato agar was prepared according to Dobereiner (1980) but malate was omitted and the sugar concentration increased to 100 g per l.

Vials with diluted cane juice medium usually showed nitrogenase activity (C_2H_2 reduction) from dilutions 10^{-2} to 10^{-6} or 10^{-7} after 3 to 7 days and characteristic growth patterns in nitrogenase positive vials were light coloured medium with whitish surface pellicles or growth throughout the medium with gas production. Such vials were replicated into semisolid LGI medium, or in later investigations, into acetic LGI medium and those showing the typical heavy orange-yellow surface pellicles on colourless medium were streated out on LGI plates. After 7 to 10 days typical dark orange colonies were transferred again into acetic LGI medium and then purified on potato agar plates.

With these methods isolates of the new bacterium were obtained from root samples of five cultivars collected from the sugarcane region of the

Table 1. Origin of strains of the new bacterium obtained from roots and stems of sugarcane in various regions in Brazil

Region	State	Sample	Number of isolated strains[a]	Sugarcane cultivars
Traditional sugarcane areas in North East Brazil	Alagoas	Washed roots	5[b]	RB 724454, RB 72102, RB 70194 CO 997, CB 45-3
	Pernambuco	Washed roots	5	CB 47-15, CR 51-22, CB 45-3
		Washed stems	5	RB 12-954, SP 70-1143
New intensively cultivated sugar cane areas in South East Brazil	São Paulo	Washed roots	5	SP 701143, CP 4374, CB 4135 NA 5779, SP 711406
		Washed stems	2	
	Minas Gerais	Washed roots	1	Native variety

[a] Many more isolates were obtained but only isolates from different samples were considered strains. All strains were identified by their characteristic growth patern in N-free semi-solid acetic LGI medium (pH 4.5) and by their dark orange-red colonies on LGI plates and brown colonies on potato medium with 10% sugar.

[b] Five samples, one from each of 5 cultivars were collected except Minas Gerais where there was only one root sample.

State of Alagoas, from all root and stem samples from five cultivars from the sugarcane region of Pernambuco; from 5 root samples and 2 stem samples from 5 cultivars collected in Sáo Paulo State and from one cultivar collected from a farm in Minas Gerais (Table 1). Four to 20 strains were then chosen for further study.

Six experiments were performed to study the effects of various carbon sources and sugar and buffer concentrations on nitrogenase activity and pH changes and to study the effects of the initial pH on N_2-dependent growth and nitrogenase activity. In all these experiments 5 ml of semisolid LGI medium in 10 ml serum vials was used as basic medium. For comparison type strains of *Frateuria aurantia* (ATCC 33424); *Gluconobacter oxidans* (ATCC 19357) and *Acetobacter aceti* (ATCC 15973), kindly provided by Dr J De Ley, were included in some experiments.

C_2H_2 reduction was estimated by closing the vials of semisolid medium with rubber stoppers and incubating under air with 10% C_2H_2 for one hour (unless otherwise stated). C_2H_4 concentration was estimated using a Perkin Elmer F11 gas chromatograph fitted with a hydrogen flame ionization detector.

All biochemical tests for the characterization of the new bacterium were performed as recommended in Bergey's Manual of Systematic Bacteriology (1984) for the characterization of the genera Frateuria, Gluconobacter and Acetobacter.

Results

In diluted cane juice medium microbial growth was observed up to dilutions 10^{-7}, occasionally 10^{-8}, but C_2H_2 reduction was observed only up to dilutions 10^{-5} or 10^{-6}, occasionally 10^{-7}. Growth in LGI medium, or in acetic LGI medium and several modifications of it, was observed only at much lower dilutions. Nitrogenase positive vials with these N-free media showed a thick yellow surface pellicle and the medium was decolourized. Most successful isolation was obtained however by transferring growth from diluted cane juice medium (light-coloured vials with whitish surface pellicles) into acetic LGI medium and subsequently streaking out on LGI or potato agar plates and incubating for 7 to 10 days. In some cases the new bacterium was also obtained from vials showing growth throughout the medium and gas production, but no other aerobic or microaerobic N_2-fixing bacteria could be isolated. No attempts were made to isolate facultative anaerobic bacteria.

On isolation plates the only colony type which consistently showed nitrogenase activity when transferred into semisolid LGI or acetic LGI medium, were dark orange to red colonies on LGI plates. These could be minute (< 1 mm) when close to colonies of other bacteria, or larger (2–3 mm, irregular) when in pure or almost pure culture. In semisolid LGI or acetic LGI media these colonies started growth after one to two days in a veil-like pellicle similar to that of azospirilla in semisolid malate medium, but with pronounced acid production. The pellicle soon concentrated on the surface and became dark yellow, growing thicker and assimilating the bromothymol blue from the medium which became colourless. These isolates in pure culture formed irregular smooth flat, initially small, white colonies which continued growing, becoming yellow, orange and finally almost red and 2–3 mm in diameter on LGI plates. The colonies became chocolate brown after 10 days on potato agar.

Preliminary attempts to isolate the new bacterium from roots and stems of sweet sorghum failed, but the characteristic growth pattern in semisolid LGI medium has been observed previously in *Azospirillum amazonense* counts with root samples of various other cereals.

The peculiar pH requirements were studied in more detail. In the study of the effect of the initial pH of semisolid LGI medium (acidified with H_2SO_4) after two days of incubation the organism showed some N_2-dependent growth with nitrogenase activity at an initial pH of 3.8 to 5.8 (Fig. 1A). This initial pH however changed rapidly, dropping to a value of around 3.0, independent of the initial pH (Fig. 1B). Much faster and more acid-tolerant growth, with an optimum at pH 3.9, was obtained when acetic acid was used to acidify the medium or when yeast extract was added (Fig. 1A). When N_2-dependent growth, evaluated by C_2H_2 reduction, was estimated later (72 h) the optimum initial pH was 6.0 because the pH of the medium after 72 h had dropped further (Figs. 1A and 1B).

In a second experiment glucose (2.5%) and sucrose (10%) were contrasted at two buffer con-

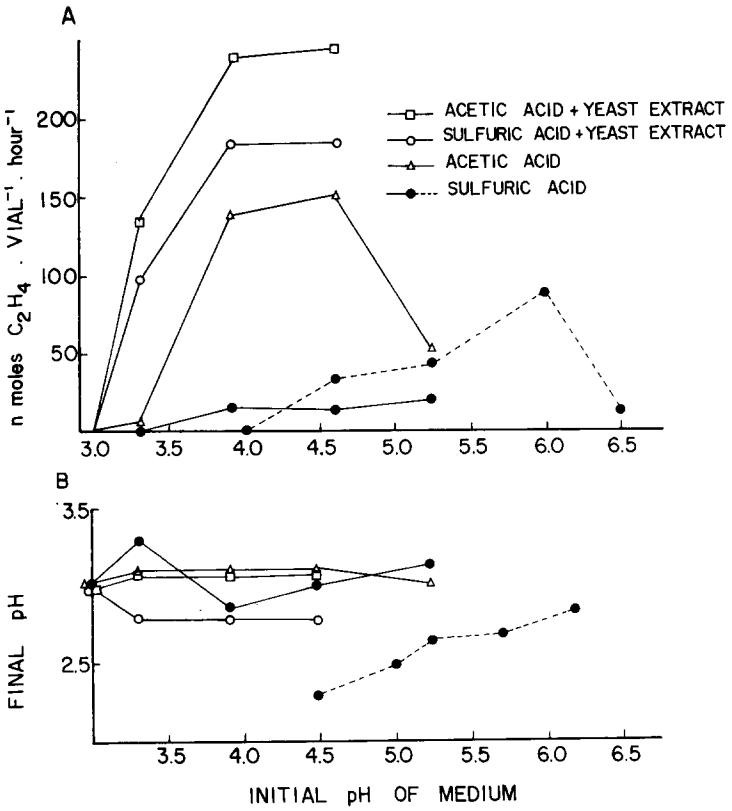

Fig. 1. Effect of the initial pH of the medium on the nitrogenase activity (Fig. 1A) and on the final pH (Fig. 1B) of the new bacterium. Semisolid LGI medium (5 ml vial^{-1}) acidified with either H_2SO_4 or with acetic acid, with or without 0.07% yeast extract, were inoculated with 0.1 ml of log phase cultures and C_2H_2 reduction measured after 48 h under air after a 1 h incubation. For comparison results from a second experiment with LGI medium acidified with H_2SO_4, incubated for 72 h, (*broken lines*) are included. Values are means of 4 strains, one vial each.

centrations (5.6 m*M* and 14.3 m*M* in phosphate) with an initial pH of 6.0 (Fig. 2) and the three acetic acid bacteria were included for comparison. No growth was observed in N-free media with these three genera and no nitrogenase activity occurred even in cultures where 0.07% yeast extract had

Table 2. Analysis of variance of the results in Fig. 2B

Source of variation	Degrees of freedom	Variance ratio (F)[a]	
		pH	Nitrogenase activity
Carbon source	1	4.15	12.70**
Buffer conc.	1	63.04**	28.89**
Time	3	34.20**	20.91**
Carbon source × Time × Buffer conc.	3	1.80 ns	2.94*
Error	45		

[a] Only data for significant effects and interactions shown.
*, ** significantly different at 5% and 1% respectively.

Table 3. Effect of sucrose concentrations on nitrogenase activity (in moles $C_2H_4 \cdot h^{-1} \cdot 5$ ml culture^{-1}) of the new bacterium in semisolid LGI medium (pH 5.5). Data are means with their standard deviations of 20 strains in Exp. I and of 4 strains in Exp. II

Concentration of sucrose[b] (%)	Experiment I[a]	Experiment II
1	62 ± 10	–
2	95 ± 16	–
8	127 ± 17	–
10	116 ± 17	31 ± 1
15	–	15 ± 7
20	–	12 ± 4
25	–	7 ± 2
30	–	14 ± 4

[a] Acetylene reduction assayed after 3 days of incubation at 31 C during 4.5 hour in exp. I and 1 hour in exp. II.
[b] Crystallized cane sugar was used.

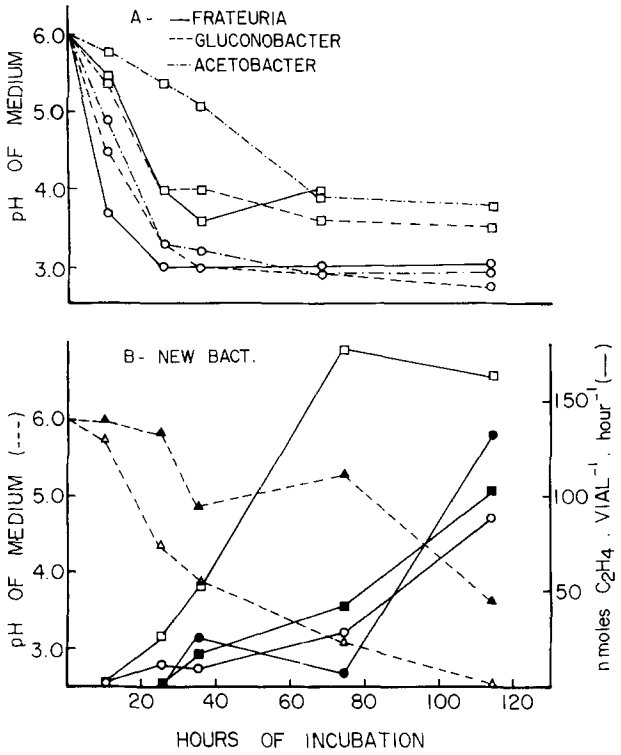

Fig. 2. Acid production from glucose or sucrose and nitrogenase acitivity of the new bacterium in comparison with three acetic acid bacteria. Acid production of *Frateuria aurantia, Gluconobacter oxidans* and *Acetobacter aceti* (Fig. 2A) and acid production and nitrogenase activity of the new bacterium (Fig. 2B) were followed in semisolid LGI medium (5 ml vial^{-1}) with initial pH 6.0 at two buffer concentrations (*open symbols* 5.6 and *closed symbols* 14.3 m*M* in phosphate respectively). For the three acetic acid bacteria the medium contained 0.07% yeast extract. Inoculation was with 0.1 ml of log phase cultures and C_2H_2 reduction was measured under air after 1 h incubation. Different vials were used for each time interval and values are single observations of the acetic acid bacteria (Fig. 2A) and means of two replicate vials from two strains of the new bacterium (Fig. 2B). Square symbols are sucrose and round symbols glucose. Triangles represent means of sucrose and glucose for pH (Fig. 2B).

been added for growth. With glucose, acid production of these three genera was faster than that of the new bacterium but all levelled out at a pH between 2.8 and 3.0. Acid production from sucrose was less active, especially with Acetobacter (Fig. 2A). Nitrogenase activity of the new bacterium (grown without yeast extract) increased during 3 days while the pH dropped from 6.0 to 3.0 and continued at increasing rates during a further 2 days with the pH at 3.0 or below. Statistical analyses of the data from the new bacterium only (Table 2) indicate significant differences between the two sugars for nitrogenase activity and for pH. With sucrose the higher buffer concentration significantly decreased nitrogenase activity, possibly because the pH remained higher (Fig. 2B).

In a third experiment the acidification of semisolid LGI medium with 1% mannitol, glycerol or ethanol was studied (Fig. 3). No acid production occurred with mannitol but there was a drop from pH 5.5 to 4.7 with glycerol. Acid production from ethanol was much faster and more pronounced and the pH dropped to 2.8 in 48 h but returned afterwards to almost the original pH (Fig. 3).

Requirement for high sugar concentrations was investigated in two additional experiments. N_2-dependent growth and nitrogenase activity occurred between 1 and 30% cane sugar with an optimum between 10 and 15% (Table 3). Visual observations at various glucose concentrations (0.1, 0.2, 0.5, 1.0, 5, and 10%) showed optical N_2-dependent growth with 5 and 10% glucose.

With such peculiar characteristics the new organism seemed to have no relationship to any known N_2-fixing bacterium but rather resembled the acetic acid bacteria even though they never have been described as being able to fix N_2. All further characterization was therefore conducted according to the differential characteristics between the three known genera of acetic acid bacteria (Bergey's Manual of Systematic Bacteriology 1984) and in comparison with one type strain each of

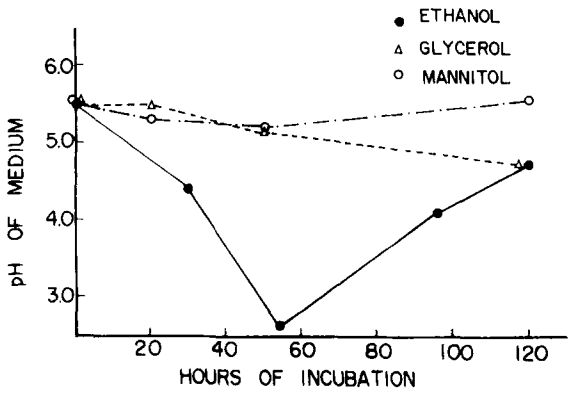

Fig. 3. Acid production and consumption from mannitol, glycerol and ethanol by the new bacterium. Vials of semisolid LGI medium (5 ml vial^{-1}), without sugar, with initial pH 5.5 and 1% mannitol, glycerol or ethanol were inoculated with 0.1 ml of log phase cultures. Different vials were used for each time interval and the values are means of 4 strains, one vial each.

Table 4. Comparison of the new bacterium with closely related genera of acetic acid bacteria as described by Swings *et al.* (1984), De Ley and Swings (1984) and De Ley *et al.* (1984)

	New bacterium	Frateuria	Gluconobacter	Acetobacter
Flagellar arrangement				
polar	−[a]	+	+	−
peritrichous	−	−	−	+
lateral	+	−	−	−
Overoxidation of ethanol	+	−	−	+
Overoxidation of glucose	+	−	−	−
Oxidation of DL-lactate to CO_2 and H_2O	+	+	−	+
Dark brown colonies on potato agar with 10% sugar	+	−	−	−
Brown water soluble pigment on GYC agar[b]	+	+	−	−
Growth factors required in mannitol medium	−	−	+	D
Formation of H_2S	+	+	−	−
Growth with 30% glucose	+	+	−	−
Growth on LGI plates	+	−	−	−
Growth in Frateur's Hoyer mineral mannitol medium	+	+	−	−
Growth on GYC[b] agar pH 4.5	+	+	+	+
N_2 fixation	+	−	−	−
NO_3-reduction	−	−	−	−
N_2 fixation with NO_3^-	+	−	−	−
Catalase	+	+	+	+
Oxidase	−	−	−	−

[a] (+) positive, (−) negative, D differs between species.
[b] Glucose yeast extract $CaCO_3$ agar plates (Micales *et al.*, 1985).

Frateuria aurantia, Acetobacter aceti and *Gluconobacter oxidans*. The results are summarized in Table 4 and a more detailed comparison with Frateuria in Table 5.

In addition to the characteristics given in Tables 4 and 5 the following descriptive observations help to characterize the new bacterium in comparison with Frateuria, Gluconobacter and Acetobacter. The new bacterium when transferred into semisolid LGI or acetic LGI medium grew readily using N_2 as the sole N source and cultures showed nitrogenase activity as evaluated by the C_2H_2 reduction method. Growth started in baloon or veil-like pellicles indicating aerotaxis and continued for more than one week when a thick surface pellicle formed which continued fixing N_2 even after the pH had dropped to 2.5 or less. The aerotactic growth pattern was most pronounced in semisolid medium with 1% of ethanol and was also observed when 10 m*M* of NH_4CL, which repressed nitrogenase, were added to the medium. Growth and nitrogenase activity were unaffected by 10 m*M* NO_3^-. In liquid medium there was no N_2-dependent growth or nitrogenase activity but there was turbidity with no surface pellicle in liquid medium with NH_4 salts or yeast extract. No growth of *Frateuria aurantia, Gluconobacter oxidans* or *Acetobacter aceti* occurred in semisolid acetic LGI medium and there was no nitrogenase activity in this medium even when yeast extract was added as N source for growth (Fig. 2A).

Most remarkable were the colonies on yeast extract-ethanol (1%) $CaCO_3$ agar and yeast extract glucose (1%) $CaCO_3$ agar recommended by Micales *et al.* (1985), for the identification of acetic acid bacteria and to which bromothymol blue had been added (3 ml 1% KOH sol per l). On the ethanol plates the new bacterium showed growth similar to Acetobacter, turning the medium first yellow and later (after 3 to 5 days) returning to blue with blue-green dry colonies while Acetobacter colonies were smooth and blue. The medium tur-

ned blue with nacreous lustre due to precipitation of the dissolved $CaCO_3$. Clear zones around isolated colonies later became white. The same plates inoculated with *Frateuria aurantia* or *Gluconobacter oxidans* remained yellow with yellow or light greenish growth. On glucose plates the new bacterium showed a growth pattern similar to that on ethanol but turned the medium dark brown after two weeks. Neither *Acetobacter aceti* nor the two other genera showed this kind of 'overoxidation' of glucose and plates even after 2 weeks remained yellow with yellow growth. On potato agar (with 10% sugar but not with 1%) the new bacterium formed very characteristic dark-brown colonies, often with lighter colour margins, while *Gluconobacter oxidans* and *Acetobacter aceti* formed white colonies on potato agar and Frateuria did not grow on it. On LGI agar plates these three genera did not grow.

Table 5. Comparison of the use of carbon substrates by the new bacterium with that of *Frateuria aurantia* according to Swings *et al.* (1984) and own observations

	New bacterium	*F. aurantia*
Growth on C sources:		
sucrose, D-galactose	++[a]	+−
D-arabinose, D-fructose	++	+−
acetate lactate	+−	+
malate	+−	+−
ethanol (1%)	+	+−
mannitol, glycerol	+	+−
i-inositol	+	+−
yeast extract	++	+
maltose	+−	+
D-mannose	++	−
Acid production from:		
sucrose, galactose	+	+
D-fructose	+	+
glucose	++	++
maltose	−	+
D-mannose	++	+
D-arabinose	++	+
ethanol (1%)	++	+
i-inositol	−	+
D-mannitol	−	−
glycerol	+	−

[a] ++ abundant growth or acidified to below pH 4.5, + good growth or acidified to below pH 5.9, +− scant or very slow growth, − no growth or acid production.

Discussion

Here we report the isolation of a new and entirely different N_2-fixing bacterium which occurs in high numbers in sugarcane roots and stems, and shows optimal growth with 10% sugar and pH around 5.5, precisely the conditions prevailing in sugarcane juice. This discovery will hopefully contribute to the elucidation of the mechanisms involved in diazotrophic associations in sugarcane and open up new pathways towards the partial replacement of nitrogenous fertilizers in a crop of great economic importance.

The new bacterium not only seems best adapted to the sugarcane environment, it is also an efficient, microaerobic N_2-fixing bacterium which is able to fix N_2 in the presence of nitrate, which may lead to the most important possibility of the complementation of nitrogen fixation with fertilizer applications. Among the N_2-fixing bacteria known so far, only *Azotobacter paspali*, which specifically associates with *Paspalum notatum* (Dobereiner, 1966; Dobereiner and Day, 1975), and *Bacillus azotofixans* (Seldin *et al.*, 1984) have been shown not to possess nitrate reductases.

According to Bergey's Manual of Systematic Bacteriology (1984) the new bacterium has to be identified within Section 4: Gram-Negative Aerobic Rods and Cocci. It seems very different from all other N_2-fixing bacteria but in view of its capacity to oxidize ethanol to acetic acid, its extreme acid tolerance, and its preference for high sugar concentrations it seems most closely related to one genus of the Pseudomonadaceae (Frateuria) and to Acetobacteriaceae (Table 4). Media acidified with acetic acid to pH 4.5 as in our acetic LGI medium have been suggested for the isolation and identification of acetic acid bacteria (Micales *et al.*, 1985). The characteristic overoxidation of ethanol which is considered the main differential characteristic of the genus Acetobacter would place it into this genus. However, several other features of the new bacterium resemble Frateuria: the brown diffusible pigment produced from glucose in the presence of yeast extract; the lack of requirement for growth substances; the formation of H_2S; and the growth in the presence of 30% glucose. Neither Frateuria nor any other members of the Acetobacteriaceae have been found to grow on N-free media or to fix N_2 in the presence of yeast extract. None of them has 1 to 3 lateral flagella. Furthermore there have been no reports of aerotactic growth patterns in

semisolid media, of the "overoxidation" of glucose or the formation of dark-brown colonies on potato agar or dark-orange colonies on LGI plates containing bromothymol blue.

No determinations of % G + C or DNA/rRNA or DNA/DNA homology studies have as yet been performed with the new bacterium. In both rRNA superfamilies (sensu De Ley, 1978), superfamily IV which includes Acetobacteriaceae and II which includes Frateuria, there are N_2-fixing bacteria (Azospirillum, Rhizobium, Xanthobacter and Beijerinckia in superfamily IV and Azotobacter and Azomonas in family II — De Smedt *et al.*, 1980; De Vos, 1980; Swings *et al.*, 1980). However, these N_2 fixers have little similarity with the new bacterium except that they fix N_2.

In view of the importance of this bacterium and the important differences which distinguish it from all possibly related bacteria, we propose a new genus and species for it which we name *Saccharobacter nitrocaptans.*

The preliminary description of this organism is as follows: *Saccharobacter nitrocaptans* gen. nov. sp. nov. (L.n. Saccharum sugar; L. bacterium) sp. nov. (L.n. nitrum; L.v. captura to put in accessible form); M.L. part adj. nitrocaptans dinitrogen fixing, straight rods about 0.7 × 2 μm rounded ends, gram negative, motile by 1 to 3 lateral flagella. Microaerobic dinitrogen fixers which grow on N_2 as sole N source in semisolid media and after initial growth with starter nitrogen in liquid media. Grow well in liquid media with combined N sources. Nitrate is not reduced and there is N_2 fixation at high nitrate concentrations (10 m*M*). Possess a respiratory metabolism with oxygen as the terminal electron acceptor. Optimum growth temperature is around 30°C and optimum pH around 5.5. Do not grow at pH 7.0 but do grow and fix N_2 at pH below 3.0. Catalase positive and oxidase negative. H_2S is formed from cystein. Growth is poor on organic acids such as malate, succinate, acetate, citrate or lactate, but acetate and lactate are oxidized to CO_2 and H_2O. 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 sucrose. Ethanol (1%), mannitol and glycerol are also used for growth. Acid is produced from ethanol and some from glycerol. On GYC plates the acid produced from ethanol and glucose is further oxidized to CO_2 and H_2O (overoxidation) and $CaCO_3$ is precipitated showing a nacreous lustre. The habitat is roots and stems of sugarcane.

Addendum

While in press, DNA/RNA TM values and DNA/DNA binding values obtained by M. Gillis (pers. comm.) showed the organism to be a new species of the genus Acetobacter. The proposed name has therefore to be changed to *Acetobacter nitrocaptans.*

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