New Nitrogen-Fixing Bacteria in Association with Non-Legumes

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Since the rediscovery in 1975 /DÖBEREINER and DAY/ of Spirillum lipoferum as a very common root-associated diazotroph, almost all basic research and most inoculation experiments have used one strain of Azospirillum brasileose, Sp7 /or Cd which is a red mutant of the same strain/ and many conclusions based on results with this strain have been widely generalized. Few results are available on experiments with other A. brasilense or A. lipoferum strains and none on the new diazotrophs which associate with grasses and cereals. No unequivocal proof of N_2 fixation in economically important amounts due to inoculation with diazotrophs is as yet available. But several data obtained from experiments without inoculation give clear evidence that N_2 fixation associated with Gramineae can be significant. A typical example is that of rice, reported by APP et al. /1984/ where total N analyses in long-term fertility plots at two sites in the Philippines, performed before and after 17 and 24 crops, yielded positive balances of 103 and 79 kg N/ha/year. Estimates over shorter periods were obtained by $^{15}N_2$ incorporation or ^{15}N dilution methods. Substantial, though very variable amounts of N_2 fixation have been demonstrated with these methods in rice /ESKEW et al., 1981; WATANABE et al., 1987/, sorghum /GILLER et al., 1984/, forage grasses /DE-POLLI et al. 1976/ and sugar cane /LIMA et al., 1987/. In order to exploit all these naturally occurring associations and improve them, the responsible diazotrophs must be identified and the mechanisms of their interactions with plants be better understood.

In this paper we will summarize and discuss the most important characteristics of several new diazotrophs which associate with cereals and sugar cane and bring additional evidence for substantial N_2 fixation in such systems.

New root-associated diazotrophs

A number of additional root-associated diazotrophs were isolated on N-free media indicating that the localization of such bacteria is on sites where O_2 is limited. A comparison of the most important microaerobic diazotrophs is given in Table 1.

Table 1

Comparison of microaerobically N_2-fixing bacteria which occur in association with plant roots /TARRAND et al. 1978, SELDIN et al. 1984, BALDANI et al. 1986, CAVALCANTE and DÖBEREINER, 1988, REINHOLD et al. 1987/

	Azospirillum brasilense A. lipoferum	A. amazonense	A. halopraeferans	Herbaspirillum seropedicae	Acetobacter diazotrophians	Bacillus azotofixans
Growth under air	+	+	+	+	+	+
Microaerobic N_2-fixation	+	+	+	+	+	−
Growth with N_2 as sole N source	+	+	+	+	+	+
N_2 fixation unaffected by 10 mmol NO_3^-	−	−	−	−	+	+
Use of sucrose	−	+	−	−	+	±
Optimum pH	6.0-7.0	5.8-6.6	6.8-8.0	5.3-8.0	3.8-5.5	6.5-7.5
Optimum temperature /°C/	35	35	41	35	30	32
Isolated from surface-sterilized roots	+	+	−	+	+	+
Isolated from stems	+	+	−	−	+	−

The two new Azospirillum spp., A. amazonense /MAGALHÃES et al. 1983/ and A. halopraeferans /REINHOLD et al. 1987/, are genotypically close and distinct from the two classical species, although some of their physiological characteristics are opposite A. amazonense is acid-tolerant and uses sucrose, being frequently isolated from sugar cane and sugar sorghum roots. It occurred in 53% of a large number /345/ of forage grass root samples and 32% of surface sterilized root samples but its numbers were smaller than those of classical azospirilla /MAGALHÃES et al. 1983/. It has also been found in roots of palm trees in the Amazon region /MAGALHÃES and DÖBEREINER, 1984/. A. halopraeferans is common in salt-affected soils in Pakistan /REINHOLD et al. 1987/. The organism shows a remarkable adaptation to this environment with a temperature optimum for growth and N_2 fixation of 41 °C and a salt requirement of 0.25% /Fig. 1/. The species was not found in salt-affected soils in Rio de Janeiro or in the semiarid region of North East Brazil, but A. brasilense strains isolated from roots or grasses grown in these soils showed a considerable increase in heat and salt tolerance as compared with the Sp7 type strain /Fig. 1/. None of these isolates showed an optimum temperature for N_2 dependent growth at 41 °C but some of the strains isolated in the North East showed a still significant ARA at this temperature. All isolates and the Sp7 type strain were able to grow at this temperature with combined N, some of them only with a slight increase in doubling time /REINHOLD et al., 1988/.

A less acid-tolerant diazotroph was found to predominate in maize roots in Brazilian Savanna soils /cerrados/ and was later isolated from washed and surface-sterilized roots of maize, rice and sorghum in Rio de Janeiro. It was initially identified as a fifth Azospirillum species but later RNA/RNA hybridization studies showed it to be a new genus, named

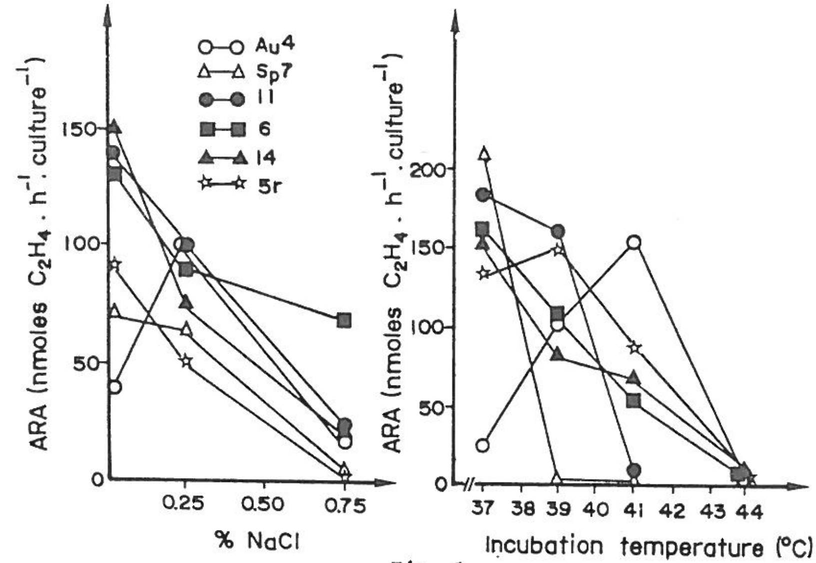

Fig. 1

Tolerance to salt and high temperatures in <u>Azospirillum halopraeferans</u> /Au4/, <u>A. brasilense</u> ATCC 29145 /Sp7/ and three <u>A. brasilense</u> /11, 6, 14/ and one <u>A. lipoferum</u> /5r/ strains isolated from the semiarid North East of Brazil /REINHOLD et al., 1988/

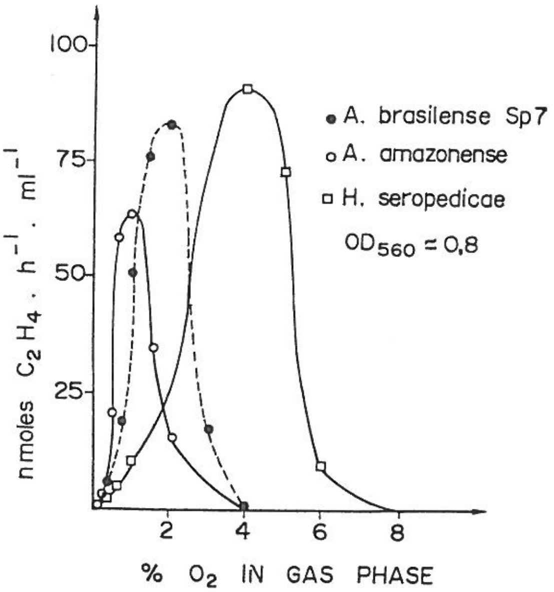

Fig. 2

Oxygen tolerance of nitrogenase activity in <u>Herbaspirillum seropedicae</u>, in comparison with <u>Azospirillum brasilense</u> and <u>A. amazonense</u> /BALDANI et al., 1986; MAGALHAES et al., 1983/

Herbaspirillum seropedicae /BALDANI et al. 1986/. The organism has bipolar flagella, and N_2 fixation is more O_2- and pH-tolerant than that of azospirilla /Fig. 2/. The nitrogenase activity of this organism and of A. amazonense is only partially inhibited by 10 mmol NH_4^+ over several hours, while that of A. brasilense and A. lipoferum is completely inhibited within minutes /HARTMANN et al. 1986, FU et al. 1988/. These authors attributed the lack of the NH_4^+ switch-off mechanism observed in most other diazotrophs, to a non-covalent inhibitory mechanism resulting in incomplete inhibition of nitrogenase activity.

Bacillus azotofixans /SELDIN et al. 1984/, a much more efficient facultative anaerobic N_2-fixing bacterium than other Bacillus spp., was isolated from surface-sterilized roots of grasses, wheat and sugar cane. In contrast to other Bacillus spp. it is capable of N_2-dependent growth and also of N_2 fixation in the presence of NO_3^-.

Acetobacter diazotrophicus isolated from sugar cane /CAVALCANTE and DÖBEREINER, 1988, GILLIS et al. 1989/ is the most recently discovered diazotroph. This most extraordinary diazotroph was originally isolated from semi-solid sugar cane juice inoculated with dilutions of sugar cane roots and stems which showed ARA up to dilutions 10^{-6} or 10^{-7}. Improved counting and isolation rpocedures were obtained in N-free mineral medium containing 10% cane sugar and 0.5% cane juice, acidified with acetic acid to pH 5.5. The bacterium is a small Gram-negative aerobic rod showing pellicle formation /microaerobic chemotaxis/ and ARA even in N-free semi-solid medium without cane juice, forming a thick surface pellicle after 5 days. Best growth occurs with high sucrose or glucose concentrations /10%/, and strong acid production results in a final pH of 3.0 or below. Growth and N_2 fixation /more than 100 nmoles/h.ml/ continues at this pH for several days /STEPHAN et al. 1988/. Ethanol is used for growth and is oxidized to CO_2 and H_2O. Dark brown colonies form on potato agar with 10% sugar and dark orange colonies on N-poor /0.005% yeast extract/ mineral agar medium with 10% sugar and bromothymol blue. The bacterium posesses no nitrate reductase and N_2 fixation is not affected by high levels /25 mmol/ of NO_3^-. Also, NH_4^+ causes only partial inhibition of nitrogenase /TEIXEIRA et al. 1987, FU et al. 1988/. This type of NH_4^+ regulation of nitrogenase, which was also observed in Azospirillum amazonense and Herbaspirillum seropedicae, leads to the suggestion of 2 categories for the NH_4^+ inhibition of nitrogenase activity: a/ covalent modification of the Fe protein by gln and/or a derivative; b/ no direct effect of Fe protein where NH_4 may affect the electron donation pathway to nitrogenase /FU et al., 1988/. The incomplete inhibition of N_2 fixation by NH_4^+ in these organisms, as well as the lack of nitrate reductase cited above, are of considerable ecological and agronomic importance because they may permit complementation of BNF with N fertilizers.

Taxonomic studies based on DNA and rRNA analyses showed that the bacterium belongs to the Acetobacter rRNA cistron /GILLIS et al., 1988/ and is most similar to A. liquefaciens. This species, however, does not fix N_2, does not form pigmented colonies on potato media and shows several other physiological differences /Table 1 and 2/. DNA/DNA binding experiments confirm it to be a new species /GILLIS et al. 1988, 1989/. Therefore, the name originally proposed /Saccharobacter nitrocaptans; CAVALCANTE and DÖBEREINER, 1988/ had to be changed to Acetobacter diazotrophicus.

The bacterium has been found in many sugar cane cultivars in several regions of Brazil and numbers were in the range /all per g wet weight/ of 10^3 to 10^5 in rhizosphere soil, 10^3 to 10^7 in washed roots, 10^3 to 10^5 in surface-sterilized roots, 10^3 to 10^6 in basal and apical stems and 10^4 to 10^7 in sugar cane trash /DÖBEREINER et al. 1988/. It was not found in the

Table 2
Comparison of Acetobacter diazotrophicus with similar Acetobacteriaceae
/CAVALCANTE and DÖBEREINER, 1988; GILLIS et al., 1988, 1989/

	A. diazotrophicus	A. aceti	A. liquefaciens	Gluconobacter oxidans
Flagellar arrangement				
polar	–	–	–	+
lateral	+	+	+	–
Overoxidation of ethanol	+	+	+	–
Oxidation of DL lactate to CO_2 and H_2O	+	+	+	–
Dark brown water-soluble pigment on GYC agar	+	–	+	–
Pyrones from D fructose	+	–	+	+
5-ketogluconic acid from D glucose	–	+	d	d
2.5-diketogluconic acid from D glucose	+	–	+	–
Growth with 30% glucose	+	–	–	–
N_2 fixation and growth on it	+	–	–	–
NO_3- reductase	–	–	–	–
Ubiquinone type	Q10	Q9	Q10	Q9
Mol% G+C of DNA	61-63	56-60	62-65	57-64
DNA/DNA homology with 3 strains of A. diazotrophicus	>90	<10	<10	<10

d - Variable

soil between the rows or on the roots of 12 different weed species growing there. Nor was it found in grain or sugar sorghum, but was isolated from a few samples of washed roots and aerial parts of Pennisetum purpurem cv. Cameroon.

Recently, Acetobacter diazotrophicus was isolated from roots, tubercles and stems of sweet potato, another plant propagated vegetatively by cuttings. When micropropagated sterile plantlets were inoculated with A. diazotrophicus by immersion of the rootlets into a culture prior to acclimatizing and transplanting into fumigated soil inoculated with MVA mycorrhiza, pronounced effects on plant growth and N incorporation were observed /Table 3/. Non-mycorrhizal plants did not grow at all. Even though no definite data are yet available to prove that the almost doubling of N% in roots plus tubercles and tops was due to BNF, it seems difficult to explain the enhanced N assimilation by hormonal effects and it cannot be attributed to the bacterial nitrate reductase as shown in some Azospirillum brasilense strains in association with wheat /FERREIRA et al. 1987/, because Acetobacter diazotrophicus does not possess a nitrate reductase /CAVALCANTE and DÖBEREINER, 1988/. Inoculation with A. diazotrophicus also increased the % MVA infection and phosphate assimilation in these sweet potato plants /PAULA et al. 1989/. On the other hand, mycorrhization seemed to enhance infection of the potatoes with Acetobacter, which would be isolated from the leaves of these plants only in the MVA treatments /PAULA et al. 1989/.

Table 3

Effect of inoculation with Acetobacter diazotrophicus on yield and N incorporation of sweet potatoes grown from micropropagated sterile plantlets transferred into fumigated soil inoculated with MVA /means of 6 replicates/ /after PAULA et al., 1989/

Treatment	N% roots	N% tops	Total-N mg/2 plants	Tuber yield g/pot
20 ppm N				
Control	0.57c	1.07c	225b	13.5c
A.diazotrophicus + Klebsiella sp.	0.93a	1.81b	848a	32.8a
A.diazotrophicus type strain Pal3	0.95a	1.74b	710a	25.8ab
60 ppm N				
Control	0.84b	1.83c	693ab	17.7bc

A. diazotrophicus and Klebsiella sp. isolated from sweet potato.

Potential of BNF in grasses and sugar cane

The potential for N_2 fixation in forage grasses such as Brachiaria spp. /BODDEY and VICTORIA 1986/ and Panicum genotypes /MIRANDA and BODDEY 1987/ has now been confirmed with the ^{15}N isotope dilution method. Depending on the plant genotype, up to 40% of the total N incorporation can come from

Table 4

Estimates of BNF contributions /kg/ha/year/ in 9 sugar cane varieties grown in a tank of 15-N labelled soil. Means of 4 replicates /S. URQUIAGA, R. M. BODDEY and J. DÖBEREINER, in press/

Variety	Estimates of BNF contributions			
	^{15}N method* First year		Total N difference** Mean of 3 years	
	%	kg/ha	%	kg/ha
CB 47-89	42	111	46	95
CB 45-3	44	113	60	164
NA 56-79	43	106	43	83
IAC 52-150	46	125	45	89
SP 70-1143	52	128	57	148
SP 71-799	46	112	42	80
SP 79-2312	42	84	48	102
Chunnee	37	56	0	0
Krakatau	56	163	68	233

*Estimate obtained with the 15-N isotope dilution technique using Brachiaria radicans as the non-fixing control plant.

**Estimate obtained from difference in total N accumulation between the sugar cane variety and the B. radicans control.

biological N_2 fixation and plant breeding aimed at increased BNF has already started. The most impressive data on the scope of plant genotype effects on BNF have been found with sugar cane cultivars /Table 4/. This crop can produce 200 tons of sugar cane which incorporate more than 250 kg N/ha every year. Acetobacter diazotrophicus has been found in high numbers /up to 10^7/ all along the canes. These characteristics, together with the highly efficient C-4 photosynthetic pathway indicate this crop as the best potential candidate for biomass production in the replacement of fossil fuels.

The recent advances in N_2 fixation research in the tropics indicate many new possibilities for the replacement of nitrogen fertilizers and for increases in crop yields. Biotechnology will play a major role in speeding up progress and helping to solve problems which, with traditional selection or adaptation methods, would take much longer to be solved.

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