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Identification and Ecology of *Herbaspirillum seropedicae* and the Closely Related *Pseudomonas rubrisubalbicans*

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Abstract

Ecological distribution of *Herbaspirillum seropedicae* in sorghum, sugarcane and forage grasses was investigated using a specific medium (JNFb). This bacterium was isolated from roots and aerial parts of various gramineous plants but not from uncropped soils. *H. seropedicae* inoculated (10^8cel·g^{-1}) into non-sterilized soil maintained at 65% of field capacity, did not survive for 21 days. Morphological and physiological tests showed that *H. seropedicae* and *Pseudomonas rubrisubalbicans* strains become elongated in glucose medium with NH_4Cl but not with malate, and this is suggested as a parameter to differentiate strains of *H. seropedicae*/*P. rubrisubalbicans* from other diazotrophs. Both species can tolerate up to 100 g·l^{-1} sucrose under nitrogen-fixing conditions at 30 or 34°C. Growth, but no nitrogen fixation, occurred at 38 and 41°C only in the presence of an organic nitrogen source. Nitrogen fixation ability of *P. rubrisubalbicans* and *H. seropedicae* was confirmed by $^{15}N_2$ incorporation into the cells.

Keywords: *Herbaspirillum*, cell morphology, ecology, diazotrophs, *Pseudomonas rubrisubalbicans*

1. Introduction

Studies on the ecological distribution of *Herbaspirillum seropedicae* have been restricted to information published on the characterization of this genus

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(Baldani et al., 1986). The lack of a specific medium to distinguish this microorganism from *Azospirillum* spp. has contributed to the dirth of information accumulated in the last few years. It has been shown that the genus *Herbaspirillum* has many similar characteristics to the genus *Azospirillum*, including the ability to grow on semi-solid NFb malate medium, (Baldani et al., 1986) a medium commonly used for the isolation and identification of the latter. In addition, very recently it was found that the plant pathogen which causes mottled stripe disease in sugarcane, known as *P. rubrisubalbicans* (Hale and Wilkie, 1972), is not *Pseudomonas* and has a high degree of DNA:rRNA homology to *Herbaspirillum* (Gillis et al., 1990). Even more interestingly this species, erroneously classified as *P. rubrisubalbicans* (Goor et al., 1986), was able to grow and fix nitrogen in N-free NFb malate medium and showed many physiological characteristics similar to *Herbaspirillum* (Pimentel et al., 1991). Thus, the development of a selective medium became important to evaluate the ecological distribution of these organisms in the soil community and in association with plants as well as to identify characteristics to differentiate this group from *Azospirillum* spp.

The purpose of this paper is to report the ecological distribution of *Herbaspirillum* in sorghum, sugarcane and forage grasses by using a more selective medium and to present additional information with respect to similarities and differences between this species and *P. rubrisubalbicans*.

2. Materials and Methods

Isolation procedures

A N-free semi-solid modified NFb medium (Döbereiner, 1980) was elaborated to study the ecological distribution of *Herbaspirillum seropedicae* in soil and roots of various plants. The changes were based on known physiological characteristics of the genus and consisted of a decrease of the pH level to 5.8, three fold increase of the phosphate concentration and omission of vitamins. This new medium (JNFb) permitted the detection of relatively high numbers of these bacteria during the survey studies realized.

The procedure used for isolation and identification of this organism was basically the same as applied to *Azospirillum*. Vials with semi-solid JNFb medium were inoculated with serial dilutions of soils, rhizosphere soils and roots of several plants as well as stems and leaves of sugarcane, and incubated for 1 week at 32°C. Vials in which a fine white pellicle developed were examined under the microscope, and transfers were made to a new semi-solid JNFb medium. After incubation for 24 to 36 hr at 32°C, they were streaked onto plates of JNFb agar containing 20 mg/l of yeast extract. On these plates, small moist

colonies with green centers developed after 5 days; *Azospirillum* colonies on such plates are white, round and dry. Phase contrast microscopy showed that *H. seropedicae* has curved rods (0.6–0.7×4–6 μm) with typical spirilloid movement, when they are close to air bubbles. *A. brasilense* cells are much wider (1 to 1.2 μm) and in young cultures much more motile. *A. amazonense* shows intermediate cell diameter (0.8 to 1.0 μm) with pronounced PHB granules. Colonies are wrinkled, small and inbedded in the agar.

Survival in soil

Survival studies of *Herbaspirillum seropedicae* strains Z67 and ZM176, isolated from rice and maize roots, resp., were carried out in non-sterilized soil maintained at 65% field capacity. Plastic pots containing 50 g of a red-yellow podzolic soil were inoculated with 0.2 ml of cultures containing 10^9 cells/ml of strains grown in liquid JNFb medium with NH_4Cl (20 mM). The control treatment received only distilled water. Survival was determined by counting and reisolation on semi-solid JNFb medium 0, 1, 2, 4, 7, 14 and 21 days after incubation at 30°C.

Morphological and physiological tests

Cell morphology of *Herbaspirillum seropedicae* and *P. rubrisubalbicans* strains was compared in semi-solid JNFb medium containing malate or glucose as the sole carbon sources and with or without NH_4Cl (20 mM). The cells were examined under the microscope 24 to 48 hr after incubation at 32°C.

H. seropedicae Z67 (ATCC 35892) and *P. rubrisubalbicans* M_4 (ATCC 19308) were tested for their ability to grow and fix nitrogen in semi-solid NFb medium with malate, containing different sucrose concentrations (0, 50, 100 and 150 $g \cdot l^{-1}$) at four temperatures (30, 34, 38 and 41°C). After 24 and 48 hr of incubation, the nitrogenase activity was evaluated under 10% V/V acetylene. A Perkin Elmer, poropak N column chromatograph was used to determine ethylene formation.

$^{15}N_2$ incorporation

To confirm the nitrogen-fixing capability of strains misnamed as *P. rubrisubalbicans*, an experiment with enriched $^{15}N_2$ was performed under laboratory conditions. Strains of this group and of *H. seropedicae* were grown in small vials with semi-solid JNFb medium for 72 hr at 32°C. Vials were sealed and 10% of the gas phase was replaced with $^{15}N_2$. Vials were incubated for 8 hr at 32°C and analyzed for total nitrogen and ^{15}N enrichment as described by Boddey et al. (1983).

3. Results and Discussion

Ecological aspects

The main problem faced during studies on the ecological distribution of *Herbaspirillum seropedicae* seemed to be solved with the semi-solid JNFb medium. With this medium we were able to detect higher numbers of *H. seropedicae* than *Azospirillum* spp. using MPN counts, from the same sample. The highest MPN dilution vials showing bacterial growth frequently contained this organism only, while *Azospirillum* spp. appeared in the lower dilution vials (Table 1). The ecological distribution of this genus in soil and several plants is also given in Table 1. Isolates were obtained in JNFb medium from rhizosphere soil and washed and surface sterilized roots of the forage grasses *Pennisetum purpureum*, *Digitaria decumbens*, *Brachiaria* sp. and *Melinus minutiflora*, however, no *H. seropedicae* was isolated from *Paspalum notatum* cv. *batatais*. This bacterium was also isolated from washed and surface sterilized roots of

Table 1. Ecological distribution of *H. seropedicae* in various gramineous plants

Plants	Source	*Azospirillum* obtained from dilution	*Herbaspirillum* obtained from dilution*
Sorghum	washed roots	10^{-3}	10^{-5}
	steril. roots[a]	10^{-3}	10^{-4}
Pennisetum purpureum	rhiz. soil[b]	10^{-4}	10^{-4}
	washed roots	10^{-3}	10^{-5}
	steril. roots	10^{-4}	10^{-5}
Brachiaria decumbens	rhiz. soil	10^{-3}	n.i.
	washed roots	10^{-3}	10^{-3}
	steril. roots	10^{-3}	n.i.
Digitaria decumbens	rhiz. soil	10^{-3}	10^{-3}
	washed roots	10^{-2}	10^{-3}
	stril. roots	10^{-2}	10^{-4}
Melinus minutiflora	rhiz. soil	10^{-3}	10^{-3}
	washed roots	10^{-3}	10^{-3}
	steril. roots	10^{-3}	n.i.
P. notatum cv. *batatais*	rhiz. soil	10^{-2}	n.i.
	washed roots	10^{-3}	n.i.
	steril. roots	10^{-3}	n.i.
sugar cane	rhiz. soil	10^{-3}	10^{-3}
	washed stems	10^{-3}	10^{-4}
	washed leaves	10^{-2}	10^{-5}

n.i. — not isolated
* — identified by light microscopy and isolation on JNFb plates
[a] — 5 min in chloramine-T (1%)
[b] — Rhizosphere soil

Table 2. Occurrence of *Herbaspirillum seropedicae* in uncropped soils and weed plants in JNFb medium

Samples	pH	Total no. diazotrophs/ g soil or roots	Presence of *H. seropedicae*
soil 1	4.5	95	–
soil 2	4.9	65	–
soil 3	5.3	140	–
soil 4	5.2	n.d.	–
soil 5	5.0	n.d.	–
weed roots[a]		250	+
weed roots[b]		1.2×10^5	+

n.d. — not detected
[a] — weed plant grown on soil 5
[b] — weed plant grown between rows of sugarcane

sorghum, confirming previous results on its occurrence in association with cereals (Baldani et al., 1986). Occurrence of *H. seropedicae* was also observed in roots, stems and leaves of various sugarcane varieties grown in different regions of Brazil, confirming previous data on the presence of *H. seropedicae* in leaves and stems of sugarcane (Pimentel et al., 1991). Recent results have also shown the varieties collected from different regions of Brazil (unpublished). An interesting aspect of the ecological distribution of *H. seropedicae* was its absence from soils maintained without crops and free of roots for 10 months (Table 2). Nevertheless, it could be isolated from roots of one unidentified weed plant grown in one of the collected soils (Table 2). In contrast, *Azospirillum brasilense* was present in low numbers (10^2cells/g soil) in some of the soils tested. *H. seropedicae* was also found in roots of weed plants grown between rows of sugarcane (Table 2), but it was not found in soil free of roots.

These results reinforce the hypothesis that bacteria of the genus *Herbaspirillum* could be plant-endophytes similar to the nitrogen-fixing bacteria, *Acetobacter diazotrophicus* (Döbereiner, 1992). The mechanisms involved in the colonization of these plants, especially cereals, are not known, although there may be a role of VA mycorrhiza in plant infection in the same way as with *A. diazotrophicus* (Paula et al., 1991). Another possibility could be transmission through seeds because this organism has been occasionally found in seeds (de Oliveira, Olivares and Baldani, unpublished data).

The absence of *H. seropedicae* in soil led us to speculate about its survival in soil without plants. The results shown on Fig. 1 indicate a decline in the population of both strains of *H. seropedicae* inoculated into soil maintained at 65% field capacity. After 21 days the initial number of 10^8 cells per g soil was

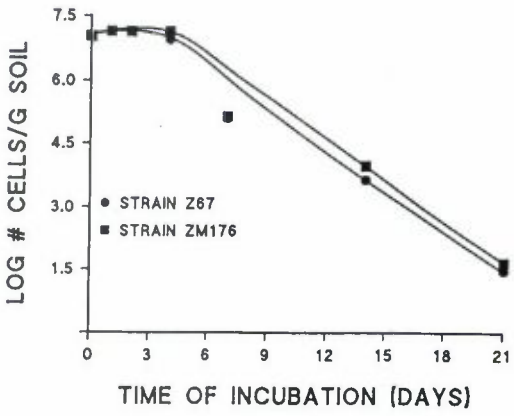

Figure 1. Survival of *Herbaspirillum seropedicae* strains in non-sterilized soil maintained at 65% of field capacity. No *H. seropedicae* could be isolated from the control vessels. Data are means of two replications.

reduced to less than 100. These results support the data on Table 2 indicating that *H. seropedicae* does not survive well in soil without plants.

Morphological and physiological aspects

In addition to the ecological studies, morphological and physiological tests were carried out to differentiate *H. seropedicae* and *P. rubrisubalbicans.* A very distinct characteristic not reported before and which seems to be a good parameter to differentiate *Herbaspirillum seropedicae/Pseudomonas rubrisubalbicans* from other diazotrophs (e.g. *Azospirillum* spp.) is cell elongation in semi-solid glucose medium in the presence of NH_4Cl (20 mM). A slight difference between *H. seropedicae* and *P. rubrisubalbicans* was observed on this medium. The latter became more elongated and thinner while *H. seropedicae* appeared elongated and wider. Elongated cells were also observed with mannitol, glycerol, galactose and arabinose but not with dicarboxylic acids such as: malic, succinic, citric, keto-glutaric and fumaric (data not shown). The cause of the elongation is unknown, although it appeared to be related to bacterial cell division and changes of pH, which increase with the use of carboxylic acids and decrease when sugars are used.

The effects of sucrose concentration and temperature on growth of *H. seropedicae* and *P. rubrisubalbicans* on semi-solid NFb medium are given on Table 3. Both groups tolerate and fix nitrogen at up to 100 $g \cdot l^{-1}$ of sucrose at 30 and 34°C. Optimum growth temperature was 30°C for *P. rubrisubalbicans* and 34°C for *H. seropedicae.* However, the strains were able to grow, but not fix nitrogen, at 38 and 41°C in the presence of combined nitrogen (20 ppm yeast

Table 3. Effect of sucrose and temperature on the nitrogenase activity of *H. seropedicae* Z67 and *P. rubrisubalbicans* M_4 in semi-solid JNFb medium

Strains	Sucrose level ($g \cdot l^{-1}$)	ARA (nmoles $C_2H_4 h^{-1}$ culture^{-1})[a]							
		24 hours				48 hours			
		Temperature (°C)							
		30	34	38	41	30	34	38	41
Z67	0	157	189	0	0	150	19	0	0
	50	36	155	0	0	126	2	0	0
	100	72	139	0	0	106	16	0	0
	150	12	1	0	0	4	2	0	0
M_4	0	96	42	0	0	260	6	0	0
	50	80	30	0	0	151	2	0	0
	100	60	11	0	0	118	3	0	0
	150	2	4	0	0	16	2	0	0

[a] — Data are means of two replications

Table 4. $^{15}N_2$ incorporation into cells of *P. rubrisubalbicans* and *H. seropedicae* strains grown in semi-solid JNFb medium

Strains	% ^{15}N excess
P. rubrisubalbicans	
M1 (LMG 1278)	0.5271
M4 (ATCC 19308)	0.4891
M5 (LMG 6415)	0.5681
M6 (LMG 6420)	0.5172
IBSBF 175 (LMG 10462)	0.3881
H. seropedicae	
Z67 (ATCC 35892)	0.5881
Z78 (ATCC 35893)	0.4405
ZM 176	0.4891
Controls	
M4 + 20 mM NH_4^+	0.0000
Z67 + 20 mM NH_4^+	0.0000

[a] — Data are means of three replications

extract, data not shown). Ability to grow and fix nitrogen in the presence of high concentrations of sucrose suggests an osmotolerance of both bacteria similar to that observed for *A. diazotrophicus* (Hartmann et al., 1991).

Although *P. rubrisubalbicans* has shown many characteristics similar to *H. seropedicae* including the ability to reduce acetylene (Pimentel et al., 1991), no data were so far available demonstrating $^{15}N_2$ incorporation by this group of bacteria. The results presented in Table 4 show incorporation of $^{15}N_2$ into the cells by five strains of *P. rubrisubalbicans* and three strains of *H. seropedicae* and therefore, confirm the nitrogen-fixing capacity of these mildly phytopathogenic bacteria.

In conclusion, the results show that *H. seropedicae* is not restricted to cereals, but was also found in association with several forage grasses and sugarcane stems and leaves. Its absence from uncropped soil as well as its poor survival in soil suggests an endophytic habitat of the bacteria. Additional morphological and physiological tests added to the available information on these two groups of microorganisms. The utilization of rhamnose and perhaps the shape and size of the cells in glucose medium with NH_4Cl were the only characteristics so far which appear to distinguish *H. seropedicae* and *P. rubrisubalbicans.*

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