

Determination of the most frequent N_2 -fixing bacteria in a rice rhizosphere

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Representative N_2 -fixing bacteria have been isolated from the rhizosphere of rice using the "spermosphere model." They have been characterized using conventional biochemical tests, DNA composition, and DNA-rRNA hybridization studies. In addition to a large number of *Enterobacteriaceae*, the most commonly encountered isolates belong to the genus *Azospirillum* and to *Pseudomonas paucimobilis*, a taxon related to *Flavobacterium capsulatum*. A nonmotile *Azospirillum* has been found.

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Des bactéries fixatrices d'azote ont été isolées de la rhizosphère de plants de riz en utilisant le "modèle spermosphère." Ces bactéries ont été caractérisées par l'utilisation des tests biochimiques conventionnels, la composition de l'ADN et des études d'hybridation ADN-rARN. En plus des divers *Enterobacteriaceae*, les isolats les plus souvent rencontrés appartiennent au genre *Azospirillum* et à *Pseudomonas paucimobilis*, un taxon apparenté à *Flavobacterium capsulatum*. Un *Azospirillum* immobile a été isolé.

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Introduction

Recent studies of free-living, N_2 -fixing bacteria associated with the roots of higher plants show similar results. A background of facultative anaerobes comprising *Bacillus* and *Enterobacteriaceae* (Vlassak *et al.* 1973; Kaputska and Rice 1976; Nelson *et al.* 1976; Pedersen *et al.* 1978; Watanabe 1981; Wright and Weaver 1981), is usually reported in addition to *Azotobacter* and *Azospirillum* (Yoshida 1970; Döbereiner *et al.* 1972; Döbereiner and Day 1976; Lakshmi Kumari *et al.* 1976; Vlassak and Jain 1976). Nevertheless, this uniformity perhaps reflects only the simplicity of isolation and identification procedures. It is well known, for instance, that the carbon source of the selective media conventionally used to count and isolate N_2 -fixing bacteria encourages the growth of specific bacteria. For example, the use of mannitol or glucose leads to the numeration and isolation of *Azotobacteraceae* (Thompson and Skermann 1979), while the use of malate favours the isolation of *Azospirillum* (Okon *et al.* 1977) from the root zone of plants. On the other hand, many scientists have often isolated bacteria difficult or impossible to identify. Very few of them have published those findings and given a precise description of isolates

or attempted to maintain them in culture. Thus, many studies on the rhizospheric free-living, N_2 -fixing microflora suffer from the use of too selective media for counts and isolations and from the lack of precise identifications of isolates.

In an attempt to overcome the carbon bias of many methods for counting and isolating rhizosphere N_2 -fixing bacteria, we have recently described (Thomas-Bauzon *et al.* 1982) a method in which a germinating surface-sterilized seed provides bacteria with exudates instead of using an artificial carbon source. In a study of a rice rhizosphere we could show that the use of this technique (the "spermosphere model") induced a very high enrichment in N_2 -fixing bacteria and made their isolation easy. Representative isolates had been tentatively assigned to *Enterobacteriaceae*, *Azospirillum*, and *Pseudomonas* after some biochemical and morphological tests. However, the considerable controversy about N_2 -fixing *Pseudomonas* (Anderson 1955; Proctor and Wilson 1958; Paul and Newton 1961; Hill and Postgate 1969; De Ley and Park 1966), along with some unusual features of those strains, lead us to make a more precise study of their taxonomic position, using biochemical tests as well as

DNA base composition and DNA-rRNA hybridization. The purpose of this paper is to report the result of this study.

Materials and methods

Bacterial strains

The following reference strains were used: *Azospirillum brasilense* Sp7 (ATCC 29145), *A. brasilense* 51e, *A. lipoferum* Br10, and *A. lipoferum* U.S.A. 5c, which were isolated by J. Döbereiner (Tarrand *et al.* 1978) and were kindly provided by R. J. Rennie (Lethbridge, Canada); *Enterobacter cloacae* ATCC 13047 and *Flavobacterium capsulatum* ATCC 14666 from the American Type Culture Collection; and *Klebsiella pneumoniae* M5a1 from the Pasteur Institute, Paris. *Agrobacterium tumefaciens* C58 was provided by J. Denarie (Toulouse, France).

The N₂-fixing bacteria used in our study were isolated from a rice rhizosphere (Thomas-Bauzon *et al.* 1982). The natural abundance of these microorganisms in the rhizosphere was higher than 10⁵/g dry soil. Among the 32 pure cultures we chose five representative strains identified as 1AB1, 4B, 4T, 5AJ, and 7ATr.

Identification

The strains were first studied by conventional methods used at the Pasteur Institute, in Paris (Stanier *et al.* 1966; Veron 1975), including morphological, physiological features, and resistance to some antibiotics. The gram reaction was confirmed after Cerny (1973).

The results of these tests were then compared with those obtained by the rapid procedure of API 20 B and API 20 E microtube systems (appareils et procédés d'identification. La Balme, Les Grottes, 38390 Montalieu Vercieu, France). The API 20 B system is devised for the identification of heterotrophic aerobic bacteria, while API 20 E system contains some tests specially designed for the identification of *Enterobacteriaceae* and other gram-negative bacilli.

Preparation of high molecular weight DNA

DNA was prepared according to a modification of Marmur's procedure (1961) by De Ley and Tytgat (1970). Melting points (T_m) or DNA were determined with an automatic recording spectrophotometer, and base composition was calculated by the equation of Marmur and Doty (1962) as modified by De Ley (1970). To decrease the remaining impurities (proteins, polysaccharides, extrachromosomal DNA), the DNA used for determination of the thermal stability of DNA-rRNA hybrids was purified by equilibrium density centrifugation in CsCl₂, denatured and fixed on cellulose nitrate membrane filters as described by De Ley and De Smedt (1975).

Preparation of [2-¹⁴C]uracil rRNA

Radioactively labelled rRNA was prepared by the method of De Ley and De Smedt (1975) using [2-¹⁴C]uracil as precursor. The specific activities of the rRNA's were as follows: 2267 cpm/μg for the 23S subunit of *Azospirillum brasilense* (ATCC 29145) and 4703 cpm/μg for the 16S subunit of *Flavobacterium capsulatum* (ATCC 14666).

Hybridizations

DNA-rRNA hybridizations, ribonuclease treatments, hybrid stability, and chemical determination of the DNA

content on the membrane filter were performed as described by De Ley and De Smedt (1975). Membrane filters loaded with 61, 58, and 60 μg high molecular weight single-stranded DNA of 4B, 4T, and 5AJ strains, respectively, were incubated in sodium saline citrate buffer, pH = 7.0 (1 × sodium saline citrate buffer contains 0.15 M NaCl + 0.015 M trisodium citrate) containing 20% formamide (called 2 × SSC-20 FA) and 10 μg of 2-¹⁴C-labelled rRNA. The filters were incubated in vials at 50°C for 16 h. After hybridization each filter was removed, incubated for 5 min at 50°C in 2 × SSC-20 FA buffer to remove nonspecifically bound rRNA, rinsed, and incubated in 2 × SSC solution containing the RNAase at 37°C for 1 h.

The thermal stability of each hybrid on a filter was measured by increasing the temperature in 5°C steps for 15 min from the hybridization temperature up to 90°C.

The radioactivity in each series of vials was measured in a liquid scintillation spectrometer.

Requirement for biotin in *Azospirillum*

Cultures grown overnight in nutrient broth, at 28°C with agitation, were centrifuged and washed twice in sterile water. One-tenth millilitre of the suspension diluted with 8.5% KOH to a final concentration of about 10⁷ bacteria mL⁻¹ was used to inoculate a 5-mL volume of medium of the following composition (grams per litre): (NH₄)₂SO₄, 1.0; K₂HPO₄, 0.2; MgSO₄ · 7H₂O, 0.2; FeSO₄ · 7H₂O, 0.01; Na₂MoO₄ · 0.02; CaCl₂ · 2H₂O, 0.026; and succinic acid, 5. The pH was adjusted to 7.0 with 1 N KOH, and the medium was autoclaved at 110°C for 20 min. After cooling to room temperature filter-sterilized biotin (0.0001 g L⁻¹) was added. Where slight growth occurred in the medium without biotin, another transfer was made to media with and without biotin. Cultures were incubated at 37 and 28°C for 2 days.

Acidification of carbohydrates by *Azospirillum*

Preparation of the bacteria was as described above; a 50-μL inoculum suspension of each strain (10⁸ bacteria mL⁻¹) was added to each of three wells of a microtiter plate, followed by 300 μL of medium. One microtiter plate containing 96 wells was used for each type of carbohydrate. The composition of the medium was as follows (grams per litre): K₂HPO₄, 0.2; FeSO₄ · 7H₂O, 0.01; Na₂MoO₄ · 2H₂O, 0.001; (NH₄)₂SO₄ · H₂O, 0.002; MgSO₄ · 7H₂O, 0.2; NaCl, 0.1; CaCl₂ · 2H₂O, 0.026; (NH₄)₂SO₄, 1.0; biotin, 0.0001; yeast extract (Difco), 0.05; bromocresol purple (Merck) aqueous, 0.04; and agar (Difco), 15.0. The pH was adjusted to 7.1 with 1 N KOH. The sugars used had a final concentration of 10 g L⁻¹. The basal medium was autoclaved at 110°C for 20 min, and each sugar was filter sterilized. The microtiter plates were incubated at 37°C for 72 h. Carbohydrate acidification was determined to be positive when the medium turned yellow (pH < 5.0), weakly positive when it became green, and negative when it remained purple violet.

Results

Morphological and physiological characters

Tables 1 and 2 show the main results for five strains, 1AB1, 4B, 4T, 5AJ, and 7ATr, from 32 isolates compared with these from the standard biochemical classification (Buchanan and Gibbons 1974). The results suggest 5AJ strain should belong to *Pseudomonas*

paucimobilis according to Holmes *et al.* (1977). 1AB1 and 7ATr strains belong to *Enterobacteriaceae*; they are, respectively, *Klebsiella oxytoca* biotype C and *Enterobacter cloacae* biotype 47737 (Richard 1981). API determinations were in very good agreement with conventional methods in use at the Institut Pasteur. For the two remaining isolates 4B and 4T, morphological and physiological tests suggested that these strains had the same responses and should belong to *Azospirillum*. The results of the API 20 B and API 20 E systems for these bacteria were comparable with the results of the reference strains (Table 2) and indicated that 4B and 4T were related to *Azospirillum* and *Agrobacterium* genera; however, the results were sufficiently ambiguous that corroborative studies by other approaches to bacterial taxonomy were necessary.

DNA-rRNA hybridizations

It was obvious that more conventional tests were unsatisfactory for classification of the 5AJ strain. The genus *Pseudomonas*, as it has been described and defined up to now, consists of a heterogeneous group of microorganisms, some of which apparently were misnamed, in particular *Pseudomonas paucimobilis*. Bauwens and De Ley (1981), using DNA-rRNA hybridization methods, showed that the yellow-pigmented organisms such as *Pseudomonas paucimobilis* belong to the *Flavobacterium capsulatum* group. To check this, we hybridized the DNA of our 5AJ strain with [¹⁴C]uracil rRNA of *Flavobacterium capsulatum* ATCC 14666. To confirm the position of strains 4B and 4T, we hybridized the purified DNA of 4B and 4T with the [¹⁴C]uracil rRNA of *Azospirillum brasiliense* ATCC 29145. Table 3 shows the results of the hybridizations, expressed as the temperature $T_{m(e)}$ at which 50% of the hybrid was denatured, and the percentage of guanine + cytosine (mol% G + C). These data suggest that 5AJ strain belongs to the *Flavobacterium capsulatum* group and that 4B and 4T belong to the genus *Azospirillum*.

Species determination in *Azospirillum*

To determine the species of *Azospirillum* 4B and 4T, we studied only two types of phenotypic characters as described by Tarrand *et al.* (1978). First, requirement for biotin seems to be easier to evaluate at an incubation temperature of 28°C than at 37°C. Our strains, 4B and 4T, require biotin as do reference strains of *Azospirillum lipoferum* Br 10 and USA 5c. The two *Azospirillum brasiliense* Sp7 and 5le had the same growth in succinate medium with and without biotin (Table 4). For the acidification tests, *Azospirillum* 4B shows the classical responses of *Azospirillum lipoferum* and we consider it to belong to this taxon. On the other hand, none of tested carbohydrates except sorbitol were acidified by *Azospirillum* 4T, and in spite of its requirement for

TABLE 1. Morphological and physiological characters of representative strains

Strain	Shape	Size ($\mu\text{m} \times \mu\text{m}$)	Motility	Capsule	Spore	Flagella	Gram stain	Acid fast	Catalase	Oxidase	Cellulolysis	Hugh Leifson test	
												F ^a	O ^b
1AB1	Short rod	1 × 1.25	-	+ ^c	-	None	-	-	+	+	-	+	+
4B	Rod	1.25 × 2.5-5	+	-	-	Peritrichous	-	-	+	+	-	+	-
4T	Rod	1.25 × 2.5-5	-	-	-	Peritrichous	-	-	+	+	-	+	-
5AJ	Slender rod	1.0 × 2.5-3.7	+	+	-	polar	-	-	+	+	-	w ^d	-
7ATr	Short rod	0.6 × 1-2	+	-	-	polar	-	-	+	+	-	+	+

^aFermentation.

^bOxidation.

^cUnstable capsule only for strain 1AB1.

^dWeak character.

TABLE 2. API 20 B and API 20 E numerical profiles on N₂-fixing and related strains. Experiments were performed at 28°C and results expressed according to the "API system"

	Characters and corresponding values													
	API 20B						API 20E							
	GEL(1), NIT(2), ONPG(4)	SAC(1), ARA(2), MAN(4)	FRU(1), GLU(2), MAL(4)	AMD(1), RHA(2), GAL(4)	MNE(1), SOR(2), GLY(4)	URE(1), IND(2), H ₂ S(4)	VP(1), CIT(2), OX(4)	ONPG(1), ADH(2), LDC(4)	ODC(1), CIT(2), H ₂ S(4)	URE(1), TDA(2), IND(4)	VP(1), GEL(2), GLU(4)	MAN(1), INO(2), SOR(4)	RHA(1), SAC(2), MEL(3)	AMY(1), ARA(2), OX(4)
IABI	6	7	7	7	3	3	3	7	3	7	5	7	7	3
4B	6	0	3	0	0	1	7	1	0	1	1	0	0	7
4T	6	0	0	0	0	1	7	1	0	1	1	0	0	7
5AJ	6	3	7	7	1	0	5	3	2	0	1	0	7	7
7ATr	6	7	7	7	7	0	3	7	3	0	5	7	7	3
	Camargue strains													
Br10	6	0	2	0	0	1	7	1	2	1	5	0	0	7
Sp7	6	0	1	0	0	1	7	1	0	1	5	0	5	7
13047	6	7	7	6	7	0	3	7	3	0	5	7	7	3
M5A1	6	7	7	3	7	3	2	7	3	5	5	7	7	3
C58	6	0	0	0	0	0	6	1	0	1	5	0	5	6
	Reference strains ^a													

ABBREVIATIONS: GEL, gelatin liquefaction; NIT, nitrite production; ONPG, β-galactosidase; SAC, sucrose acidification; ARA, arabinose acidification; MAN, mannitol acidification; FRU, fructose acidification; GLU, glucose acidification; MAL, maltose acidification; AMD, starch acidification; RHA, rhamnose acidification; AMY, amygdalin acidification; GAL, galactose acidification; MNE, mannose acidification; SOR, sorbitol acidification; MEL, melibiose acidification; GLY, glycerol acidification; INO, inositol acidification; H₂S, hydrogen sulfide production; VP, Voges-Proskauer, acetoin production; OX, cytochrome-oxidase; URE, urease; CIT, citraconin citrate; IND, indole production; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; and TDA, tryptophane decarboxylase.

^aBr10, *Azospirillum lipoferrum*; Sp7, *Azospirillum brasilense* (ATCC 29145); 13047, *Enterobacter cloacae* (ATCC 13047); M5A1, *Klebsiella pneumoniae* (collection of Institut Pasteur); and C58, *Agrobacterium tumefaciens*.

TABLE 3. DNA base composition and $T_{m(e)}$ of the DNA-rRNA hybrids of three rhizosphere strains with ^{14}C -labelled reference rRNA from *Flavobacterium capsulatum* ATCC 14666 and *Azospirillum brasilense* ATCC 29145

Strain	Hybridization with [^{14}C]rRNA from:		
	G + C content	<i>Azospirillum brasilense</i> ATCC 29145, $T_{m(e)}$ ($^{\circ}\text{C}$)	<i>Flavobacterium capsulatum</i> ATCC 14666, $T_{m(e)}$ ($^{\circ}\text{C}$)
	mol%		
4B	67.6	80.0	nd
4T	67.6	80.3	nd
5AJ	67.4	nd	75.8
<i>Flavobacterium capsulatum</i> ATCC 14666	63.0	nd	80.0
<i>Azospirillum brasilense</i> ATCC 29145	67.5	81.5	nd

NOTE: nd, not determined.

TABLE 4. Species determination of *Azospirillum* 4B and *Azospirillum* 4T, using some physiological tests which permit the distinction between *Azospirillum lipoferum* and *Azospirillum brasilense*

	<i>A. lipoferum</i>		<i>A. brasilense</i>		<i>Azospirillum</i>	
	Br10	USA5c	Sp7	51e	4B	4T
Biotin requirement (at 28 $^{\circ}\text{C}$)	yes	yes	no	no	yes	yes
Acidification (at 37 $^{\circ}\text{C}$) of:						
Glucose	+	+	-	-	+	-
Mannitol	+	+	-	-	+	-
Sorbitol	+	+	+	+	+	+
Ribose	+	nd	weak	weak	+	weak

NOTE: nd, not determined.

It is very difficult to determine the species of this strain. For these reasons we call it *Azospirillum* sp. 4T. It is the first nonmotile *Azospirillum* which has been found.

In summary, among the 32 N_2 -fixing bacteria isolated with the "spermosphere model" technique and identified by classical methods as well as molecular hybridization in some cases, 25 *Klebsiella oxytoca* (similar to IAB1), 2 *Enterobacter cloacae* (similar to 7ATr), 3 *Pseudomonas paucimobilis* (similar to 5AJ), and 2 *Azospirillum* (4B and 4T) were found.

Discussion

Several *Pseudomonas* strains have been reported to fix N_2 (Anderson 1955; Proctor and Wilson 1958; Paul and Newton 1961; Watanabe 1981), and their assignment to *Pseudomonas* was based primarily on biochemical characters. It is now generally accepted that the traditional genus *Pseudomonas* is a very heterogeneous entity on which some light has been cast using modern methods, such as electrophoresis of total

soluble or envelope proteins (Kerstens and De Ley 1975), immunological studies of specific proteins (London 1975), or DNA-DNA hybridization studies (De Ley and Tytgat 1970). To study more distant relationships the most useful methodology is DNA-rRNA hybridization, based on the finding that rRNA genes have evolved less rapidly than most other genes (De Ley and De Smedt 1975).

Our strain 5AJ is a good example of what can be expected from the use of such methods: classical morphological characters as well as biochemical traits place it in *Pseudomonas paucimobilis* (Holmes *et al.* 1977), but based upon DNA-rRNA hybridization, strain 5AJ should be assigned to the recently redefined *Flavobacterium capsulatum* group (Bauwens and De Ley 1980).

The generalized use of DNA-rRNA hybridizations led De Ley (1981) to distinguish at least five superfamilies among the gram-negative bacteria, and it is interesting to note that *Flavobacterium capsulatum* belongs to the fourth superfamily along with other

plant-associated bacteria such as *Agrobacterium*, *Beijerinckia*, *Rhizobium*, *Rhodospirillum*, and *Azospirillum*.

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