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Azospirillum: Genetics of Nitrogen Fixation and Interaction with Plants [and Discussion]

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Azospirillum: genetics of nitrogen fixation and interaction with plants

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Bacteria of the genus *Azospirillum* are free-living diazotrophs that were isolated from the rhizosphere and from the roots of grasses. Genetic analysis of nitrogen fixation was essentially initiated in *A. brasilense* Sp7, where genetic tools and mutants are available. A DNA region covering 25 kb and containing the nitrogenase structural genes (*nifHDK*), *nifE* and another *nif* cluster has been cloned. In addition, the structural gene for glutamine synthetase, which might be involved in *nif* regulation, was cloned and sequenced. To identify bacterial genes involved in the root colonization process, DNA–DNA hybridization was performed with *Rhizobium* nodulation (*nod* and *hsn*) genes. Homology was detected in both cases and clones containing DNA homologous to *hsn* genes were isolated. *Azospirillum* contains large plasmids. Preliminary experiments suggest that the *hsn* homologous region is located on the 90 MDa plasmid of strain Sp7.

1. Introduction

Measurement of the nitrogenase activity by the acetylene reduction assay *in situ* enabled scientists to examine various ecosystems for nitrogen fixation potential. This approach confirmed that some nitrogen fixation was associated with non-symbiotic systems, in particular the zone around the roots of grasses. Among bacterial species identified, special interest focused on nitrogen-fixing spirillum-like bacteria (Döbereiner & Day 1976), which were classified later in a new genus, *Azospirillum* (Tarrand *et al.* 1978). Recent progress in the genetics of *A. brasilense* Sp7 (ATCC29145) is reported.

2. Taxonomy and physiology

The bacteria, first described in 1922 by Beijerinck and rediscovered in 1963 by Becking, were called *Spirillum lipoferum* (for review, see Elmerich 1986). Subsequent taxonomic studies led to the creation of a new genus, *Azospirillum*. This genus, defined by Tarrand *et al.* (1978), comprised two species: *A. brasilense* and *A. lipoferum*. Two new species, *A. amazonense* and *A. haloprae ferens*, were recently discovered (Magalhaes *et al.* 1983; Reinhold *et al.* 1987). The bacteria are Gram-negative aerobes, curved-rod-shaped with a polar flagellum, and contain globules of poly-betahydroxybutyrate. They have a DNA base composition of 66–71 mol % G+C.

Physiological properties of *Azopirillum* spp. were recently reviewed (Okon 1985*a*; Elmerich 1986). In general, the bacteria utilize organic acids, such as malate. Strains of *A. lipoferum* can

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utilize a variety of carbohydrates, including glucose, which is not used by *A. brasilense*. *A. amazonense* can utilize saccharose. Studies of the hydrogen metabolism in *Azospirillum* revealed the existence of an uptake hydrogenase activity. Autotrophy and methylotrophy were also demonstrated. *Azospirillum* species participate in all steps of the nitrogen cycle except nitrification; in particular, most strains are denitrifiers. Their ability to fix nitrogen in pure culture was established by the ^{15}N isotopic method. Nitrogen fixation occurs only under microaerobic conditions.

3. Genetics of nitrogen fixation

Cloning of a nifHDK cluster from A. brasilense Sp7

The model system for the organization of nitrogen fixation genes is *Klebsiella pneumoniae* (*oxytoca*) M5a1. In this bacterium, a cluster of 17 *nif* genes, localized on the chromosome and organized in 8 transcription units, has been identified (for review, see Dixon 1984*a*; Elmerich 1984). The nitrogenase structural genes, *nifHDK*, are carried on a 6.2 kb *Eco*RI fragment and the three genes are transcribed as part of a single operon. By homology with a *K. pneumoniae nifHDK* probe, a 6.7 kb *Eco*RI fragment, designated AbRI, was cloned from total DNA of *A. brasilense* Sp7 (Quiviger *et al.* 1982). Heteroduplex analysis, performed with the *nifHDK* cluster of *K. pneumoniae*, established the approximate location of the corresponding *Azospirillum nifH*, *-D*, *-K* genes on this fragment (Quiviger *et al.* 1982).

To determine the transcriptional organization of the *Azospirillum nifHDK* cluster, localized mutagenesis by Tn*5* was performed (Perroud *et al.* 1985). The methodology developed by Simon *et al.* (1983) in *Rhizobium meliloti* was applied to *Azospirillum*. These authors constructed plasmid pSUP202, a derivative of pBR325 that contained the site for mobilization (mob region) of incompatibility P type plasmids. Plasmid pSUP202 cannot replicate outside the enteric bacteria, and can be used as a suicide vehicle to introduce specific markers in a large number of Gram-negative bacteria (Simon *et al.* 1983). Tn*5* mutagenesis was performed in *E. coli* on plasmid pAB3, a derivative of pSUP202 in which the AbRI fragment was cloned (figure 1). Insertions located in different positions of the *nifHDK* region were obtained and recombined in the *Azospirillum* genome (figure 1). Insertions in the *nifH* or *nifDK* homology region led to a

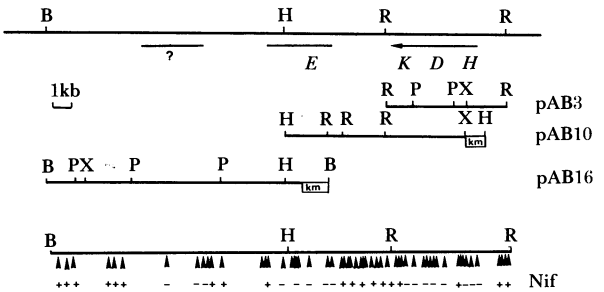

Figure 1. *A. brasilense* Sp7 *nif* clusters. *NifHDK* and *nifE* genes were identified by hybridization with *K. pneumoniae* or *Rhizobium* ORS571 *nif* probes. The arrow indicates the direction of transcription and the question mark indicates a *nif* region whose correspondence to *K. pneumoniae nif* genes has not been established. Restriction sites: B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xho*I. The vector for pAB3 was pSUP202 (Simon *et al.* 1983); the vector for pAB10 and pAB16 was pUC8 (Vieira & Messing 1982). Black triangles show Tn*5* insertions which were obtained either in pAB3 or in subclones of pAB10 and pAB16 in pSUP202. The mutated plasmids were then introduced into *A. brasilense* Sp7 and the mutation was recombined in the host genome. The Nif phenotype (+ or −) was determined by an assay of nitrogenase activity with whole cells.

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Nif$^-$ phenotype. Insertions located between *nifH* and *nifD* led to a Nif$^+$ phenotype. This observation suggested that the *nifHDK* cluster might be composed of two transcription units. However, the polarity effect of Tn*5* insertions in *nifH* on *nifD* and *nifK* was determined by genetic complementation and analysis of *nif* polypeptide synthesis. Results were in agreement with the existence of a single operon transcribed in the *nifHDK* order as in *Klebsiella pneumoniae* (Perroud *et al.* 1985).

Identification of two other nif clusters

A kanamycin cartridge, corresponding to the 2.2 kb *Xho*I fragment purified from Tn*5* and containing a single *Hin*dIII site, was inserted at the unique *Xho*I site of pAB3 (figure 1). After recombination in the Sp7 genome, the resulting Nif$^-$ strain was used to recover a 10 kb *Hin*d III fragment containing the DNA region located downstream from the *nifHDK* operon (pAB10 in figure 1). The presence of *nif* genes on this fragment was investigated by Tn*5* localized mutagenesis and another *nif* region was found. Hybridization with *K. pneumoniae* and *Rhizobium* ORS571 (Norel *et al.* 1985) *nifE* probes suggested that the newly identified *nif* region contained the equivalent of the *nifE* gene (this laboratory, unpublished). From a Tn*5* insertion located in the *nifE* homology region it was possible to recover a 15 kb *Bam*HI fragment containing the adjacent region (pAB16 in figure 1). By Tn*5* mutagenesis a third *nif* region was found (figure 1).

4. Regulation of nitrogen fixation

In enteric bacteria, the structural gene for glutamine synthetase (GS), *glnA*, belongs to a complex regulon (Merrick 1983). This regulon contains the *ntrBC* genes, the products of which, together with the product of a third regulatory gene, *ntrA*, are responsible for the transcriptional activation of a number of operons involved in nitrogen assimilation, including nitrogen fixation. Regulation of nitrogen fixation in *K. pneumoniae* involves two mechanisms (Merrick 1983; Dixon 1984*a*): (i) a *nif*-specific regulation through the products of *nifL* and *nifA*, acting respectively as repressor and activator of the other *nif* operons; (ii) a non-*nif*-specific regulation through the products of the *ntrBC* genes. In the regulation model the *ntrC* product in the presence of the *ntrA* product activates *nifA* transcription. The *nifA* product in turn, in the presence of the *ntrA* product, acts as a positive effector of the transcription of all the other *nif* operons (see Dixon *et al.*, this symposium).

Isolation of nif regulatory mutants

Some glutamine auxotrophs of *K. pneumoniae* have a Nif$^-$ or a Nif^c (i.e. fixing nitrogen in the presence of ammonia) phenotype, as a consequence of a mutation in *ntrBC* or as the result of a mis-sense or polar mutation in *glnA* (Leonardo & Goldberg 1980; Espin *et al.* 1981). *Azospirillum* contains a glutamine synthetase (GS), with features similar to the *E. coli* enzyme (see Bozouklian *et al.* 1986). *Azospirillum* mutants impaired both in GS activity and nitrogen fixation were isolated (Gauthier & Elmerich 1977). In particular mutant strains 7029 and 7028 isolated from Sp7 are Gln$^-$ Nif$^-$ and Gln$^-$ Nif^c respectively, and resemble *glnA* or *ntrBC* mutants of *K. pneumoniae* (table 1). More recently, Pedrosa & Yates (1984) isolated Nif$^-$ mutants of Sp7 whose nitrogen fixation was restored by plasmid pGE10, which contained the *glnAntrBC* regulon of *K. pneumoniae*. The authors proposed that *Azospirillum* contains genes with functions analogous to the *K. pneumoniae ntrC* gene.

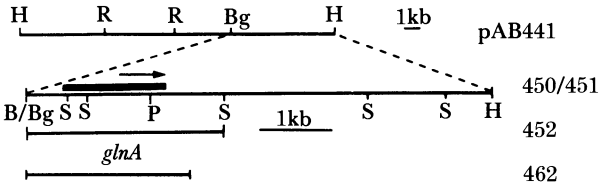

FIGURE 2. *A. brasilense glnA* region. The black rectangle indicates the position of the *glnA* gene; the arrow indicates the direction of transcription. The vector for pAB441, pAB451 and pAB462 was pVK100 (Knauf & Nester 1982); the vector for pAB450 and pAB452 was pSUP202.

TABLE 1. COMPLEMENTATION OF *A. BRASILENSE* MUTANTS

	nitrogenase activity (%†)		
	7029	7028	
	Gln^- Nif^-	Gln^- Nif^c	
NH_4^+ (20 mM)	−	−	+
no plasmid	2.7	18	200
pAB441	46	110	< 0.3
pAB451	120	70	< 0.3
pPC462	80	73	< 0.3
pPC940	30	30	0.8

† 100% corresponds to 60 nmol ethylene min^{-1} mg^{-1} protein. In the presence of NH_4, the nitrogenase activity of Sp7 is < 0.3%. The plasmid pPC940 contains the *glnA* gene from *K. pneumoniae* cloned in the broad host range vector pVK100 (Bozouklian *et al.* 1986).

Isolation of the glnA gene

A gene library of *A. brasilense* Sp7 was constructed by cloning *Hin*dIII fragments in the broad host-range vector pVK100 (Fogher *et al.* 1985). Clones were crossed with the Gln^- Nif^- mutant 7029 and complementation for prototrophy was examined. Three plasmids, containing a common 20 kilobase (kb) *Hin*dIII fragment responsible for the Gln^+ phenotype, were isolated (Fogher *et al.* 1985). Plasmid pAB441 was studied further (see figure 2 and table 1). No complementation of *E. coli* ET8051, which carries a *glnAntrBC* deletion, was observed. However, after four days, plasmid mutants were isolated at a frequency of 10^{-7}. In addition, the gene was expressed in *E. coli* when it was placed under the control of an exogenous promoter of the plasmid vector (Bozouklian *et al.* 1986).

The *glnA* gene was localized on subclones of pAB441. Tn*5* insertions and hybridization with a *K. pneumoniae glnA* probe established that the *glnA* gene was located within a 1.9 kb *Sal*I fragment (figure 2). The *glnA* product was identified as a 51 kDa polypeptide, which could be adenylylated in *E. coli* (Bozouklian *et al.* 1986).

Complementation of Klebsiella and Azospirillum glutamine auxotrophs

As reported in table 2, and in agreement with data of Espin *et al.* (1982), *K. pneumoniae glnA* mutants (e.g. UNF1787) were complemented by plasmids pGE100 and pGE102 (Espin *et al.* 1982) which both contained a functional *glnA* gene from *K. pneumoniae*. On the other hand, *ntrC* mutants were complemented only by pGE100 which also carried a *ntrBC* operon. Introduction of *Azospirillum glnA* into *K. pneumoniae glnA* mutants (e.g. UNF1787) restored a wild-type control of nitrogen fixation. In most of the *ntrC* mutants (e.g. UNF1816) no complementation occurred;

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this result suggested that the *glnA* plasmids did not contain *ntr* functions (Bozouklian *et al.* 1986).

Introduction, in *Azospirillum* Gln^- mutants, of plasmids containing *Azospirillum glnA* (pAB441, pAB451 or pAB462) (see figure 2) or *K. pneumoniae glnA* (pGE10 or pPC940) restored a wild type control of nitrogen fixation (table 1). Plasmids pAB462 and pPC940 contained only *glnA*. This suggested that strains 7028 and 7029 carried a mutation in the GS structural gene. Consequently, a direct involvement of *Azospirillum* GS in the regulation of nitrogen fixation cannot be ruled out (Bozouklian *et al.* 1986).

TABLE 2. COMPLEMENTATION OF *K. PNEUMONIAE* MUTANTS

	Nitrogenase activity (%)		
	UNF1787 *glnA*		UNF1816 *ntrC*
NH_4^+	−	+	−
no plasmid	94	42	< 0.1
pGE100	200	< 0.3	50
pGE102	53	< 0.3	< 0.1
pAB450	120	3.4	< 0.1
pAB452	295	0.5	< 0.1

The plasmid pGE100 contains the *glnAntrBC* regulon of *K. pneumoniae*; pGE102 contains only *glnA*; pGE plasmids and UNF strains are described by Espin *et al.* (1981, 1982).

Nucleotide sequence of glnA

It was of particular interest to determine the complete nucleotide sequence of *Azospirillum glnA*, because physiological evidence suggested a regulatory role of the enzyme in nitrogen fixation. In *K. pneumoniae* two promoters have been identified for *glnA* transcription. An upstream promoter, termed P1, resembles the *E. coli* canonical promoter. Transcription from P1 is repressed by the product of *ntrC* and an operator site for this repression has been identified (Dixon 1984*b*). A downstream promotor, termed P2, belongs to a class of promoters activated during nitrogen limited growth. Transcription from P2 requires the product of *ntrC* in its activator form and the product of *ntrA* (Dixon 1984*b*).

The complete nucleotide sequence of the *glnA* gene of Sp7 was established (Bozouklian & Elmerich 1986). This is the first gene sequenced from *Azospirillum*. The gene encodes a polypeptide of 468 residues of M_r 51917. The similarity coefficient between the polypeptide sequence of *Azospirillum* and *Anabaena* 7120, which is the only other *glnA* sequence available (Tumer *et al.* 1983), is 58%. The G + C content of the sequence is 64%, close to the total G + C content of *A. brasilense* estimated at 70%. The codon usage appears largely different from the common codon usage in *E. coli*, as expected from the high G + C content; for instance, 27 of the 64 coding triplets are not used.

The DNA sequence located upstream of the initiation codon was examined for transcriptional signals. No significant homology with *E. coli* canonical and *ntr* promoters, nor with the promoter region of the *Anabaena glnA* gene, was found. This suggested that, if there is *ntr*-related control in *Azospirillum* as has been proposed (Pedrosa & Yates 1984), this control should be somewhat different from the *ntr* system of enteric bacteria. Another possibility is that *glnA* escapes this control. No translation initiation site could be defined since the DNA sequence

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immediately preceding *glnA* did not show a strong homology with *E. coli* 16S rRNA terminus. However, it is worth noting that, in spite of an atypical Shine–Dalgarno sequence and a codon usage different from *E. coli*, *glnA* is well translated in *E. coli*, when it is placed under the control of the *tet* promotor of pSUP202 (Bozouklian *et al.* 1986).

5. Association with plants

Association with grasses

Azospirillum spp. were isolated from the rhizosphere of a large number of monocotyledonous and of some dicotyledonous plants (for review, see Elmerich 1984). In most cases, the bacteria were isolated after surface sterilization of the roots. No differentiated structure was formed, but it appeared that *Azospirillum* could invade the cortical and vascular tissues of the host (Döbereiner & Day 1976; Patriquin & Döbereiner 1978). After *Azospirillum* inoculation, a large enhancement of the number of lateral roots and of root hairs was observed (for review, see Okon 1985*b*). This proliferation was concomitant with an increase in mineral uptake, and was attributed to phytohormone production rather than to nitrogen fixation (Okon 1985*b*). Pictures of root hair deformation were also reported (Patriquin *et al.* 1983).

Most of the strains isolated from maize were *A. lipoferum*, and most of those isolated from wheat or rice were *A. brasilense* Nir^- (non-denitrifying), suggesting a difference of specificity between the two species towards C_4 and C_3 plants (Döbereiner & de Polli 1980). Further experiments are required, in particular with bacterial mutants, to determine the basis of the recognition process.

Homology between Rhizobium nodulation genes, Agrobacterium chromosomal virulence region and Azospirillum total DNA

Nothing is known of the genes involved in the association with the host plant. Root modifications (Patriquin *et al.* 1983; Okon 1985*b*) observed after colonization by *Azospirillum* led us to propose, as a working hypothesis, that some of the early steps in the interaction between bacteria and plants might proceed from a common mechanism (Fogher *et al.* 1985). This assumption was reinforced by the finding that *Azospirillum* could stimulate or inhibit nodulation of clover (Plazinsky & Rolfe 1985). *Rhizobium* nodulation (*nod*) genes and *Agrobacterium* chromosomal virulence (*chv*) genes are the best known examples of genetic determinants involved in bacteria-plant interaction. In *R. meliloti*, two DNA regions involved in early stages of the bacterium–plant interaction, located on a plasmid near the *nifHDK* and the *fixABC* cluster, were identified. One of them is referred to as the 'common *nod* genes' and the other as the host-specificity region (*hsn*) (Kondorosi *et al.* 1984). In *Agrobacterium* two closely linked loci, designated *chvA* and *chvB*, have been identified (Douglas *et al.* 1985).

Restricted total DNA from several *Azospirillum* strains was hybridized with common *nod* genes and *hsn* probes of *R. meliloti* and *chv* probes of *Agrobacterium tumefaciens*. Homology was detected with all probes (Fogher *et al.* 1985; Michiels *et al.* 1985). Table 3 reports the sizes of the fragments from strain Sp7 revealed by the three probes.

Cloning of Azospirillum DNA fragments homologous to Rhizobium genes

Gene banks composed of *Azospirillum* Sp7 *Eco*RI or *Sal*I fragments were constructed in pUC18. Colony hybridization was used to identify clones that carried homology to *nod*, *hsn* or

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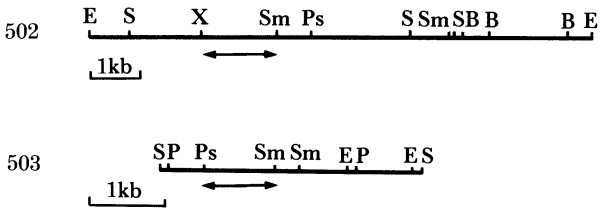

FIGURE 3. *A. brasilense* Sp7 DNA restriction fragments sharing homology with *R. meliloti* '*hsn*' region. Restriction sites: B, *Bam*HI; *E*, *Eco*RI; P, *Pvu*I; Ps, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I. Double-headed arrows show the homology region to the 1.5 kb *Eco*RI–*Sal*I fragment purified from the *hsn* region and carrying *nodG*.

TABLE 3. SIZE OF THE DNA FRAGMENTS HOMOLOGOUS TO *nod*, *hsn* AND *chv* PROBES IN *AZOSPIRILLUM* Sp7

(Sizes are expressed in kilobases (kb). Probes were as follows: *nod*, 8.5 kb *Eco*RI fragment carrying *nodDABC* from *R. meliloti* 41; *hsn*, 6.8 *Eco*RI fragment carrying *nodGFEH* from *R. meliloti* 41 (Kondorosi *et al.* 1984, 1985); *chv*, 2.3 and 6.7 kb *Bam*HI fragments containing *chvB* from *A. tumefaciens* (Douglas *et al.* 1985).)

nod		*hsn*		*chv*	
*Eco*RI	*Sal*I	*Eco*RI	*Sal*I	*Eco*RI	*Sal*I
7.2	3.3	12	5.6	7.9	11.5
		10	3.8	7.2	11
		1.8	2.4	6.9	10
				1.8	9.8
					9.6

chv. Clones containing DNA homologous to *hsn* and *chv* probes were isolated. In particular, plasmids pAB502 and pAB503, which contained fragments homologous to the *hsn* probe, were studied. Plasmid pAB502 contained a 10 kb *Eco*RI insert which carried an internal 5.6 kb *Sal*I fragment; plasmid pAB503 contained a 3.8 kb *Sal*I insert. DNA fragments cloned in pAB502 and pAB503 were used in turn to perform hybridization with the *hsn* region of *R. meliloti*. Four genes have been identified in this region: *nodG*, *nodF*, *nodE* and *nodH* (Kondorosi *et al.* 1985). In both cases the homology was detected at the level of a 1.7 kb *Bam*HI–*Sal*I fragment carrying *nodG*. Figure 3 shows the physical map of the cloned fragments and the approximate localization of the homology region.

Localization of the homology region in the host genome

In rhizobia, functions related to symbiotic nitrogen fixation were shown to be plasmid-borne. All *Azospirillum* strains examined so far contained at least one plasmid (Franche & Elmerich 1981; Plazinsky *et al.* 1983). No phenotypic property has been demonstrated as plasmid-borne. However, it is tempting to speculate that functions related to bacterium–plant associations might be present on *Azospirillum* plasmids. To determine the chromosomal or plasmidic localization of the *hsn* homologous region, Southern blot hybridization was performed. Preliminary experiments showed that the pAB502 insert hybridized with the 90 MDa plasmid contained in strain Sp7, whereas the pAB503 insert hybridized with chromosomal DNA.

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6. Conclusions

During the past ten years, a relatively large amount of information has been accumulated on the molecular biology and the genetics of *Azospirillum* as well as on the physiology of its association with the roots of grasses. In particular, progress was made on the genetics and regulation of nitrogen fixation. Another promising area is the identification of bacterial genes involved in the association with grasses. It is too early to draw conclusions on the significance of the homology detected between *nod* and *chv* genes and *Azospirillum* DNA, because we do not yet know if the corresponding regions are functional in *Azospirillum*. However, this approach should lead, in the near future, to a better understanding of the molecular basis of *Azospirillum*–plant interactions and might open the possibility of engineering associations with improved performance.

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Discussion

J. W. Drozd (*Shell Research Ltd., Sittingbourne, Kent, U.K.*). What is the current status of experiments with inoculation of crops by *Azospirillum* spp. in the field? Can *Azospirillum* contribute significantly to the nitrogen requirements of plants?

C. Elmerich. I refer this question to Dr Döbereiner, who is present.

J. Döbereiner (*EMBRAPA-UAPNBS, Seropédica, 23851 – Rio de Janeiro, Brazil*). There are several answers to this question. In the Israeli experiments, in which only one strain of *A. brasilense* (Sp7) has been used, there have been repeatable inoculation responses with various cereals. We attribute these responses to the low frequency of occurrence of *Azospirillum* spp. in

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these soils. The responses with Sp7 have been attributed to hormonal effects, enhancing root growth and thus enhancing assimilation of mineral nutrients generally.

In our experiments in Brazil we have found no inoculation responses with Sp7, probably because *Azospirillum* spp. are abundant in the soil (more than 10^5 g^{-1}). However, we have obtained, in several field experiments with wheat in the past three years, consistent increases in total plant nitrogen and especially in grain nitrogen, by inoculation with strains Sp107str. and Sp215spec. of *A. brasilense*. These antibiotic-resistance-marked strains could consistently be isolated from within surface-sterilized wheat roots, where they represented the majority of the population of *Azospirillum* spp. present. In lysimeter experiments with ^{15}N-labelled nitrogen fertilizer, inoculation of wheat with these strains enhanced assimilation of fertilizer nitrogen compared with inoculation by strain Sp7 or uninoculated controls. There was no evidence that any of the increases in plant nitrogen were due to biological nitrogen fixation in these experiments with wheat.

However, there have been a number of recent experiments showing, by ^{15}N dilution or $^{15}N_2$ incorporation, that substantial amounts of biological nitrogen fixation (up to 20–60 % of total plant nitrogen) can occur in certain sugar-cane and forage-grass varieties. In sugar cane, this was confirmed by positive total nitrogen balances after two crops. These plants had *not* been inoculated and we do not know which diazotrophic species were responsible for the observed nitrogen fixation.

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