fixA, B and *C* genes are essential for symbiotic and free-living, microaerobic nitrogen fixation

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Site-specific mutations were contructed within *Bradyrhizobium japonicum fixA, B* and *C* regions that had previously been identified by interspecies hybridization [(1985) Mol.Gen.Genet. 199, 315–322]. The corresponding mutants were not only Fix$^-$ in otherwise fully developed soybean root nodules but also Nif$^-$ in free-living microaerobic culture. Specific *fixA, B* and *C* probes hybridized to DNA of the aerobic diazotroph, *Azotobacter vinelandii*. We hypothesize, therefore, that *fixA, B* and *C* genes are of general importance for aerobic/microaerobic nitrogen fixation.

Aerobic diazotrophy (Bradyrhizobium japonicum, *Soybean)* *Symbiosis* *Mutagenesis* nif *gene* *Nitrogen fixation* fix *gene*

1. INTRODUCTION

Bradyrhizobium japonicum, a member of the slow-growing group of rhizobia, elicits the formation of root nodules on soybean (*Glycine max* L. Merr.) in which they differentiate into endosymbiotic bacteroids and are able to fix molecular nitrogen. As a strict aerobe, *B. japonicum* depends on respiration to produce ATP for growth, and for the energy-demanding nitrogenase reaction. In symbiosis, the plant-encoded leghemoglobin serves as oxygen carrier to the bacteroids [1]. In the free-living state, rhizobia are not normally able to fix N_2, with the exception of a few slow-growing strains among which is *B. japonicum* strain 110 [2]. These strains manage to fix N_2 (yet, they do not grow on N_2) at extremely low O_2 tension (microaerobiosis) that suffices to support respiration but does not destroy the oxygen-labile nitrogenase.

Several *B. japonicum* (strain 110) nitrogen fixation genes have been identified in our laboratory [3]. Some of them (e.g. the nitrogenase structural genes) have been found to be homologous to corresponding genes in *Klebsiella pneumoniae*, in which 15 functional *nif* genes are required for structure, modification, and activity of the nitrogenase complex [4]. The general agreement is that all other rhizobial symbiotic nitrogen fixation genes, which do not exhibit apparent homology to any of the *K. pneumoniae nif* genes, are to be called *fix*. The *Rhizobium meliloti fixABC* genes (forming one operon) were the first ones detected that fell into this classification [5–7]. It is not known, however, whether the *fixA, B* and *C* gene products are directly required for nitrogenase activity or, more indirectly, are required for symbiosis-specific functions in bacteroid development.

In *B. japonicum fixA-, B-* and *C*-like genes have been identified, by interspecies hybridization, on two separate genomic regions, one harboring *fixA*

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Abbreviations: nif/fix, nitrogen fixation genes; Fix$^-$, defective in symbiotic nitrogen fixation; Nif$^-$, defective in free-living nitrogen fixation; kb, kilobase pair(s)

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and the other one *fixB* and *fixC* [8]. The first aim of this work was to prove their functional necessity in symbiotic nitrogen fixation, by inserting mutations into them. The second goal was to make use of the free-living, microaerobic culture system which should allow one to test whether the respective mutants were (also) defective in N_2 fixation in the absence of the plant. Thirdly, using *B. japonicum fix* probes for hybridization, we wished to know whether these genes were characteristic to only *Rhizobium* and *Bradyrhizobium* species.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

These are listed in table 1. *Escherichia coli* HB101 was used for transformations. The use of all other strains is mentioned in the text.

2.2. *DNA biochemistry*

Cloning and analysis of recombinant DNA was performed using standard techniques [9]. The probes applied for Southern blot hybridization were ^{32}P-labelled RNA transcripts synthesized in vitro from *fixA*, *fixB* or *fixC* DNA fragments linked to the SP6 promoter of pGEM-1 [10].

Southern blots were prehybridized at 45°C for 4 h in 50% formamide, 50 mM sodium phosphate, pH 6.5, 0.8 M NaCl, 1 mM EDTA, 2.5 × Denhardt's solution [11], 20 μg/ml carrier DNA, 20 μg/ml carrier RNA. Hybridization of the blots in the presence of the RNA probes was done in the same solution at 45°C for 16–20 h. The filters were washed 4 times in 50 mM NaCl, 20 mM sodium phosphate, pH 6.5, 1 mM EDTA, 0.1% SDS at 60°C (for homologous hybridizations) or at 50°C (for heterologous hybridizations).

Table 1

Bacterial strains and plasmids

	Relevant genotypic or phenotypic characteristics	Reference or origin
E. coli strains		
HB101	*hdsR*$^-$ *hsdM*$^-$ *recA13*, Str^R	[18]
17-1	*hsdR*$^-$/RP4-2 *kan*::Tn*7* *tet*::Mu, integrated in the chromosome	[19]
B. japonicum strains		
110*spc4*	Spc^R ('wild-type')	[20]
A512	Spc^R, *fixA*::*aph* (Kan^R)	this work
A613	Spc^R, *fixA*::*aph* (Kan^R)	this work
B508	Spc^R, *fixB*::*aph* (Kan^R)	this work
C206	Spc^R, *fixC*::*aph* (Kan^R)	this work
Other bacterial strains		
Bradyrhizobium sp. 32H1	wild-type	Nitragin Co., Milwaukee, WI
R. phaseoli 8002	wild-type	[21]
P. syringae pv. *glycinea* 1883	wild-type	National Collection of Plant-pathogenic Bacteria, Harpenden, England
A. vinelandii	wild-type	Laboratory Culture Collection
Vectors and recombinant plasmids		
pGEM-1	Amp^R, SP6 and T4 promoters	Promega Biotech, Madison, WI
pSUP202	Amp^R Tet^R Cam^R, *oriT* from RP4	[19]
pEFC6	Amp^R (pACYC177), *K. pneumoniae nifJHDKYENXUSVMFLABQ*	AFRC Unit of Nitrogen Fixation, Brighton, England
p9-5B	Kan^R (pMMB33) *B. japonicum nifE fixG nifS nifB nifH fixB fixC*	[3,22] and S. Ebeling, unpublished
pRJ7107	Tet^R (pACYC184) *B. japonicum fixA*	H.-M. Fischer, unpublished

2.3. *Localized mutagenesis of the* fixA, B *and* C *genes*

The procedure results in the exchange of a wild-type genomic DNA region against the corresponding in vitro mutated DNA fragment by homologous recombination. The principle methods have been detailed [12,13] and consist of the following steps. *B. japonicum* DNA from the *fixA* or *fixBC* regions was cloned into the pSUP202 vector and mutagenized by insertion of the *Xho*I fragment from Tn*5* coding for kanamycin resistance. These recombinant plasmids were transformed into *E. coli* strain 17-1 which then mobilizes the pSUP202 derivatives into *B. japonicum* by conjugation. Kanamycin resistant, tetracycline sensitive *B. japonicum* strains were analyzed by Southern blot hybridizations of their total DNA using *fixA*-, *B*- or *C*-specific probes. True marker replacement mutants had lost their wild-type *fix* gene by double crossover.

2.4. *Assay for nitrogen fixation activity in symbiosis and in free-living culture*

The infection of soybean seedlings, growth of the plants, and measurements of N_2 fixation activity in nodules by the acetylene reduction assay were described [13,14]. Free-living *B. japonicum* cultures were grown under microaerobic conditions to derepress the genes required for N_2 fixation [15,16]. N_2 fixation activity was measured by the acetylene reduction assay.

2.5. *Electron microscopy*

17-day-old nodules from soybean plants (*G. max* var. Clark L1) infected with either the wild-type or mutant *B. japonicum* strains were cut into slices and examined by electron microscopy as described [17].

3. RESULTS

3.1. *Construction of* B. japonicum fixA, B *and* C *mutations*

The strategy of the site directed insertion and deletion/replacement mutagenesis is depicted in fig.1. Two genomic *Eco*RI fragments containing either the *fixA* gene region or the *fixB* and part of the *fixC* gene regions served as targets for the mutagenesis. The 2.35 kb *Xho*I fragment from Tn*5* carrying the *aph* gene was cloned into the

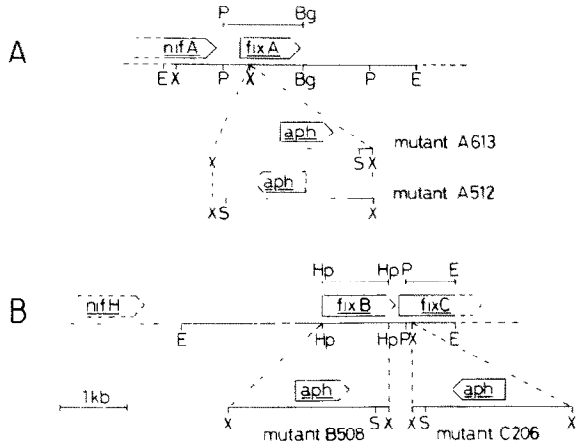

Fig.1. Constructions for the site-directed insertion and deletion/replacement mutagenesis of *B. japonicum fixA* and *fixBC* gene regions. (A) The 3.8 kb *Eco*RI fragment from pRJ7107 containing the *fixA* gene was recloned into the *Eco*RI site of pSUP202. The product was partially digested with *Xho*I and religated in the presence of the 2.35 kb *Xho*I fragment of Tn*5* which carries the aminoglycoside phosphotransferase-3′(II) gene (*aph*) specifying kanamycin resistance. The two pSUP202 derivatives with the *aph* inserts in either direction at the *Xho*I site of *fixA* were isolated and used for mobilization into *B. japonicum* resulting in the generation of mutants A613 and A512. In vitro RNA transcripts from the 1.2 kb *Pst*I-*Bgl*II fragment (drawn above *fixA*) were used as *fixA*-specific probe for the hybridization in fig.3A. (B) The 4.1 kb *Eco*RI fragment harboring *fixB* and part of *fixC* was isolated from cosmid p9-5B and recloned into the *Eco*RI site of pSUP202. The 1.0 kb *Hpa*I fragment of *fixB* was replaced by the 2.35 kb *Xho*I Tn*5* fragment whose protruding 5′-ends had been filled up by the Klenow DNA polymerase before ligation. This construct was used to generate mutant B508. The pSUP202 derivative which gave rise to the mutant C206 carries the *aph* gene inserted at the *Xho*I site of *fixC*, and was constructed in a similar way. In vitro transcripts from the 1.0 kb *Hpa*I fragment and the 0.75 kb *Pst*I-*Eco*RI fragment (drawn above *fixB* and *fixC*) served as *fixB*- and *fixC*-specific probes for the hybridizations in fig.3B,C. Restriction sites are shown for *Bgl*II (Bg), *Eco*RI (E), *Hpa*I (Hp), *Pst*I (P), *Sal*I (S), and *Xho*I (X).

genes such that the *fixA* and *fixC* genes were disrupted at their internal *Xho*I sites, and the 1.0 kb *Hpa*I fragment within *fixB* was replaced. These in vitro mutagenized DNA regions were mobilized into *B. japonicum* strain 110*spc4* in

which double crossovers between the wild-type and mutated *fix* alleles gave rise to the distinct mutants (fig.1).

3.2. *Acetylene reduction activity of free-living and symbiotically grown* B. japonicum *FixA, B and C mutations*

Soybean seedlings were inoculated with the *B. japonicum* mutants A613, A512, B508 and C206, and with wild-type strain 110*spc4* as control. All root nodules from two independent plants were harvested 15, 17 and 22 days after infection and assayed for acetylene reduction. Table 2 shows the values determined at day 22. None of the mutants showed any significant acetylene reduction activity (Fix^- phenotype).

In a second experiment, the same strains were cultured under free-living microaerobic conditions and assayed for acetylene reduction. The measured values are also listed in table 2. All mutants showed only low background activity characteristic for nitrogen non-fixing strains [16], i.e. usually less than 5% of the ethylene accumulated by the wild-type (Nif^- phenotype). After prolonged incubation, no further increase in acetylene reduction was observed in the mutant cultures, which indicates that the mutations in the *fixA, B, C* genes did not simply cause a delayed Nif^- phenotype.

Table 2

Acetylene reduction activity of *B. japonicum* FixA, B and C mutants compared with strain 110*spc4* (wild-type)

	In root nodule symbiosis[a]	In microaerobic, free-living culture[b]
110*spc4* (wild-type)	122.5	7.435
A613 (FixA mutant)	0.034	0.470
A512 (FixA mutant)	0.07	0.626
B508 (FixB mutant)	0.025	0.391
C206 (FixC mutant)	0.029	0.313
Uninfected plant	0.06	–

[a] At least 40 nodules were measured 22 days after infection. Activity is expressed as μmol $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ (nodule dry wt)

[b] Accumulation of C_2H_4 8 days after the first addition of O_2. Activity is expressed as nmol $C_2H_4 \cdot ml^{-1}$ (culture). The numbers are mean values of 4 independent cultures

3.3. *Ultrastructure of nodule cells infected with* B. japonicum *Fix mutants*

Visual examination of wild-type- and mutant-infected root nodules from 17-day-old plants revealed no differences with regard to their number, size, distribution over the root system, and the apparent content of leghemoglobin. Electron microscopic examination showed that FixA, B and C mutants are perfectly able to fully differentiate into endosymbiotic bacteroids surrounded by peribacteroid membranes. This is exemplified by fig.2 in which wild-type- (A) and FixA mutant-infected (B) nodule cells are compared. The major notable difference was that mutant bacteroids contained a significantly increased amount of poly-β-hydroxybutyrate, a rhizobial reserve material that is known to be stored when bacteroids are unable to fix N_2 [17].

3.4. *Interspecies hybridization using specific* fix *probes*

The results in preceding sections suggest that *fixA, B* and *C* genes are not specifically involved in the *B. japonicum*–soybean interaction but are important for microaerobic fixation of N_2. This prompted us to support these data by testing whether *fixA-, B-* and *C*-like genes could be present in other closely related bacteria which (i) form root nodules on legumes, or (ii) interact specifically with soybean, or (iii) fix N_2 only under free-living and obligatory aerobic conditions. For this purpose interspecies hybridizations were performed using *B. japonicum fixA-, B-* and *C*-specific probes (see fig.1 for the probes used). Fig.3 shows that all three probes hybridized to total DNA of *Bradyrhizobium* sp. 32H1 and *R. phaseoli* (lanes 2 and 3, respectively). In *R. phaseoli* the hybridizing band was always a 7.4 kb *Eco*RI fragment (lane 3) suggesting that all three genes may be adjacent just as in *R. meliloti* [7]. *Bradyrhizobium* sp. 32H1 has two *fixA-* (6.6 and 5.9 kb), one *fixB-* (6.8 kb) and two *fixC-* (6.8 and 1.25 kb) homologous bands (lane 2) suggesting that one of the genes may be separate from the others, similar to the situation in *B. japonicum* [8]. Surprisingly, all three *fix* genes hybridized (lane 4) to a 7.0 kb band within total DNA of *Azotobacter vinelandii* which is known to be unable to form specific symbiotic interactions with host plants. In contrast the soybean-specific pathogen,

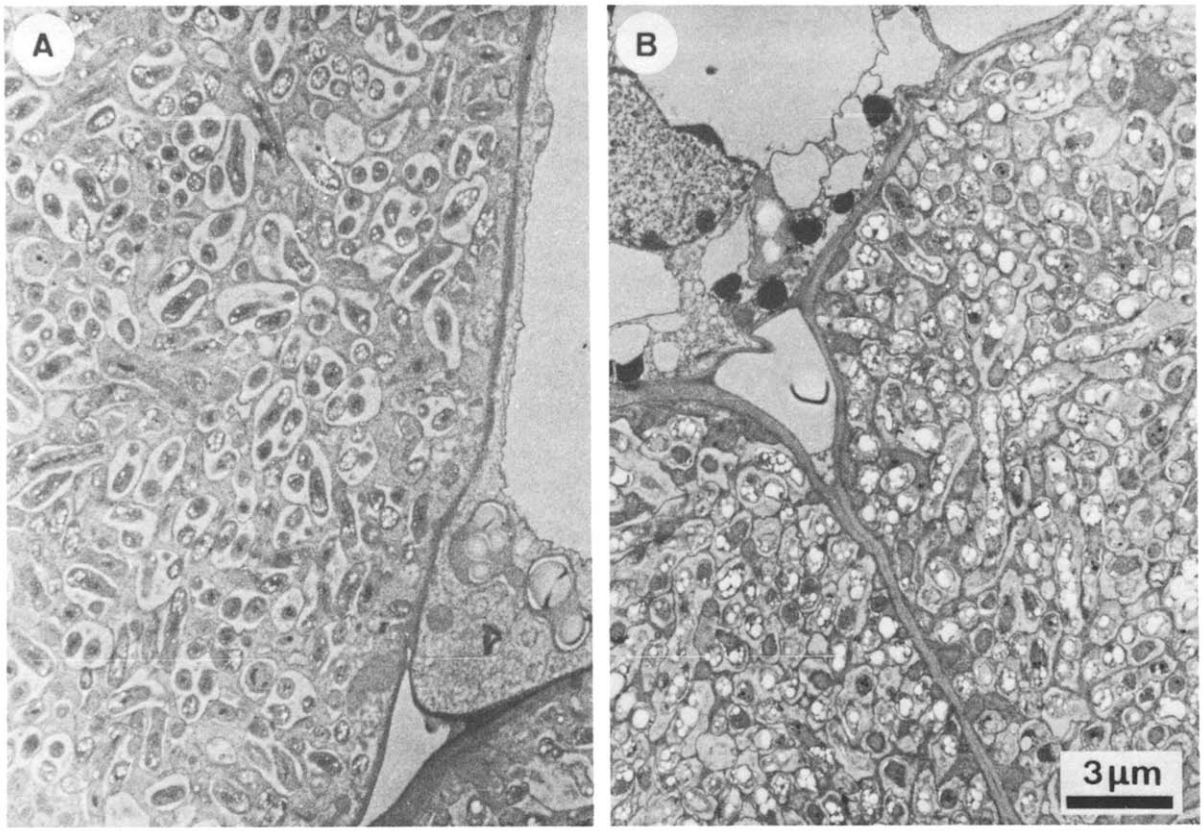

Fig.2. Electron microscopic examination of nodule cells. (A) Wild-type-infected nodule cell filled with bacteroids which contain little poly-β-hydroxybutyrate (PHB). (B) Nodule cells infected by FixA mutant A512. The bacteroids contain a large amount of PHB, recognizable as round, completely white granules. (Samples and photos prepared by D. Studer.)

Pseudomonas syringae pv. *glycinea* does not appear to carry *fix* homologous genes (lane 5). Also, *fix* genes did not hybridize to any of the *nif* genes (lane 6) in the complete *nif* cluster of *K. pneumoniae* which fixes N_2 only under fermentative, anaerobic conditions.

4. DISCUSSION

Structural and functional evidence for the existence of *fixA, B* and *C* genes is now available for *R. meliloti* [5–7] and *B. japonicum* ([8], and this work). In addition, interspecies hybridization experiments suggest that similar genes may be present in the *Sesbania Rhizobium* ORS571 [23,24], in *R. phaseoli* (this work) and in *Bradyrhizobium* sp. 32H1 (this work). Hence, *fixA, B* and *C* genes appear to be characteristic for all rhizobia. However, this work has shown that these genes are obviously not involved in functions required for bacteria–plant interaction: (i) electron microscopic analysis revealed that FixA, B and C mutants develop almost normally into mature root nodule bacteroids, similarly to mutants with defective *nifD, K, H, B* and *E* genes ([17]; S. Ebeling and M. Hahn, unpublished); (ii) the results of table 2 revealed that *fixA, B* and *C* gene products are essential for free-living, microaerobic N_2 fixation; (iii) *fixA, B* and *C* genes also appear to be present in non-symbiotic N_2 fixing bacteria such as *A. vinelandii* (this work) and *Azospirillum brasilense* [25]. Notably, all *Rhizobium, Bradyrhizobium, Azotobacter* and *Azospirillum* species are microaerobic or aerobic diazotrophs, i.e. they all depend on energy conservation by respiration using O_2 as terminal electron acceptor. The question

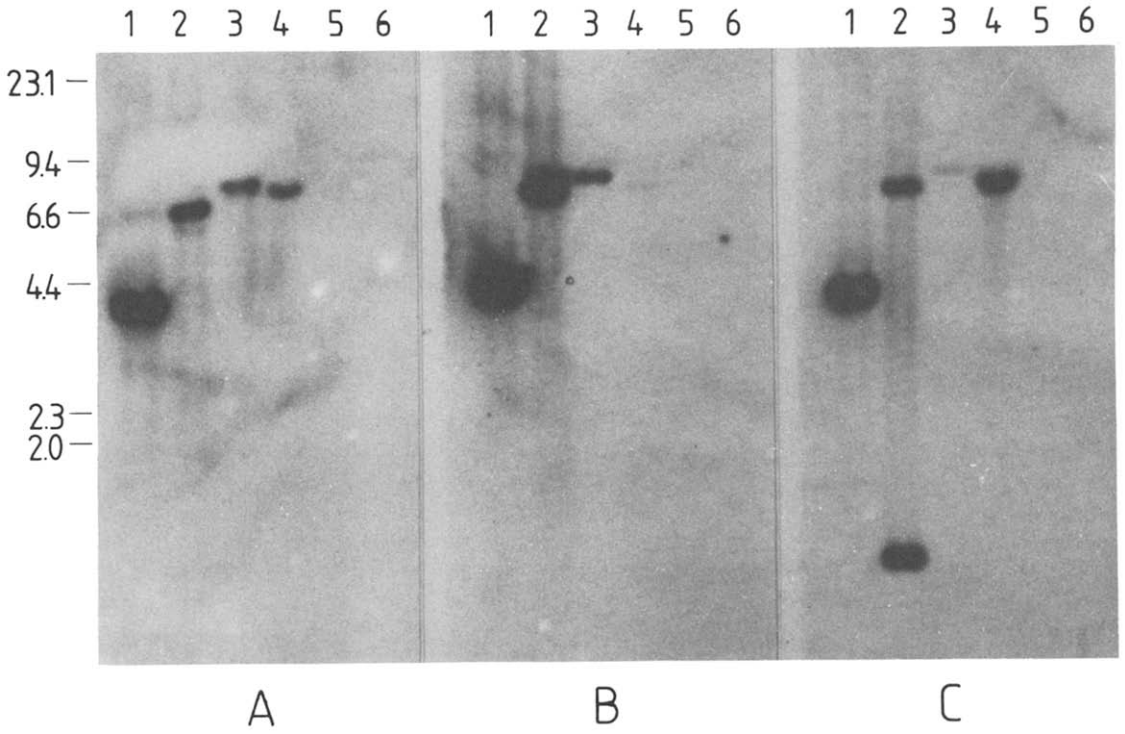

Fig.3. Interspecies hybridization using specific *fix* probes. Autoradiographs are shown of three identical Southern blots. The radioactive probes were ^{32}P-labelled in vitro RNA transcripts from subcloned *B. japonicum fix* gene fragments which are shown in fig.1. Blot (A) was hybridized with *fixA*-, blot (B) with *fixB*-, and blot (C) with *fixC*-specific transcripts. The lanes contain equal amounts (~2 μg) of *Eco*RI-digested total genomic DNA from *B. japonicum* (1), *Bradyrhizobium* sp. 32H1 (2), *R. phaseoli* (3), *A. vinelandii* (4) and *P. syringae* pv. *glycinea* (5). Lane 6 contains 50 ng of *Eco*RI/*Hin*dIII double-digested pEFC6 plasmid DNA representing the complete *K. pneumoniae nif* gene cluster.

then arises: What roles do the *fixA, B, C* gene products play in microaerobic/aerobic diazotrophy? Almost all of the 15 functional *K. pneumoniae nif* genes [4] have now been identified in either of the two diazotrophic aerobes, *A. chroococcum* [29] and *B. japonicum* ([3]; S. Ebeling and M. Hahn, unpublished), with the exception of *nifL, Q* and *J*. Are *fixA, B* and *C* functionally homologous to anyone of these genes, even though they do not hybridize (fig.3) to the complete *K. pneumoniae nif* cluster? Since *nifL* codes for a repressor [4], mutations in *nifL* would not normally be Fix$^-$ or Nif$^-$, unless such mutations are polar on other genes. *nifQ* determines an early function in the biosynthesis of the FeMo cofactor for nitrogenase [28]. Although a single gene suffices for this step in *K. pneumoniae*, one cannot completely rule out the possibility that one or all of the *fixA, B, C* gene products fulfil a similar task. *nifJ* codes for a pyruvate-flavodoxin-oxidoreductase [26]. One would not expect to find a *nifJ* analogous gene in obligatory (micro)aerobic diazotrophs, because in these bacteria the generation of reducing power for nitrogenase is believed to be linked to the respiratory chain [27], and not to a fermentation end-product such as pyruvate in *K. pneumoniae* [26]. On the basis of all these considerations we favor the hypothesis that in aerobic diazotrophs at least some, if not all, of the *fixA, B* and *C* genes code for essential functions in electron transport to nitrogenase.

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