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A New *Bradyrhizobium japonicum* Gene Required for Free-Living Growth and Bacteroid Development Is Conserved in Other Bacteria and in Plants

Marianne Weidenhaupt, Monika Schmid-Appert, Beat Thöny, Hauke Hennecke and Hans-Martin Fischer

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland.

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In the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum*, a new DNA region, *orf74*, was discovered which is required for optimal free-living growth and, by consequence, also necessary for the formation of an effective symbiosis. A Tn*5*-233 insertion in *orf74* resulted in a mutant, strain 74, that has a reduced growth rate in free-living cultures under all conditions tested and less than 1% residual symbiotic nitrogen fixation activity as compared with the wild type. Nodule distribution and nodule morphology are severely affected similarly as in a previously characterized *B. japonicum nifA* mutant. Protein databank searches revealed that the 142-amino-acid protein encoded by *orf74* is homologous to a 161-amino-acid protein encoded by *orf17* of *Bacillus subtilis* (approximately 46% identity; J. C. R. Struck, R. Kretschmer-Kazemi Far, W. Schröder, F. Hucho, H. Y. Toschka, and V. A. Erdmann, Biochim. Biophys. Acta, 1050:80-83, 1990) as well as to a 178-amino-acid protein encoded by *orf178* of *Escherichia coli* (approximately 45% identity; K. L. Poulsen, N. W. Larsen, S. Molin, and P. Andersson, Mol. Microbiol., 6:895-905, 1992). Significant similarity was also found with unknown ORFs of two plant species. When expressed from a strong constitutive promoter, *orf17* of *B. subtilis* could partially complement *B. japonicum* mutant 74. By contrast, *orf74* of *B. japonicum* was unable to functionally complement an *E. coli orf178* mutant. The conservation of this DNA region in gram-negative and gram-positive bacteria suggests that the gene is essential for a fundamental cellular process which is required in *B. japonicum* for both free-living and symbiotic growth.

Additional keywords: complementation, Gef phenotype, Tn*5* mutagenesis.

The gram-negative aerobic soil bacterium *Bradyrhizobium japonicum* is able to induce nitrogen-fixing nodules on the root of its host plant, soybean (*Glycine max* L. Merr.). The initiation as well as the maintenance of a functional symbiosis requires specific environmental conditions and the expression of a substantial number of bacterial genes involved in nodulation, bacteroid metabolism, and symbiotic nitrogen fixation (Barbour et al. 1992). In *B. japonicum*, a low cellular oxygen concentration is the essential signal for the expression of symbiotic nitrogen fixation genes. The regulatory activator proteins NifA and $FixK_2$, the latter being dependent on the two-component regulatory system FixLJ, govern two hierarchic cascades of genes (for review see Fischer 1994). Under low oxygen concentrations the two-component FixLJ system induces the synthesis of the activator protein $FixK_2$ which in turn controls the expression of genes involved in microaerobic respiration, anaerobic respiration with nitrate (Anthamatten and Hennecke 1991, Anthamatten et al. unpublished, Preisig et al. 1993), and σ^{54} ($RpoN_1$) synthesis (Kullik et al. 1991). The control of σ^{54} synthesis by FixLJ/$FixK_2$ connects this regulatory cascade to the NifA regulatory cascade in *B. japonicum* because σ^{54} is essential for the transcription from −24/−12 promoters that are associated with all known NifA-activated genes or operons (Thöny and Hennecke 1989; Merrick 1993). Among these are the nitrogen-fixation genes (e.g. *nifHDK*; Fischer et al. 1986) and genes not directly related to nitrogen fixation (e.g., *glnII*, Martin et al. 1988). In *B. japonicum,* both the synthesis and the activity of the NifA protein are subject to regulation. Control of NifA activity consists of the inactivation of the NifA protein under aerobic conditions by an unknown mechanism (Fischer and Hennecke 1987). Synthesis of *B. japonicum* NifA is regulated dually at the level of transcription (Thöny et al. 1989). Under aerobic growth conditions, the *fixRnifA* operon is expressed at a basal level. This expression is dependent on a DNA region located around position −66 upstream of the transcriptional start site and on a postulated activator protein (Thöny et al. 1989). In fact, the formation of a DNA-protein complex could be demonstrated using crude extracts of *B. japonicum* wild-type cells grown either aerobically or anaerobically and a DNA fragment spanning the *fixR*-upstream region.

Under symbiotic conditions the expression level of the *fixRnifA* operon is enhanced by the NifA protein. In an attempt to identify the gene(s) encoding the postulated activator protein essential for the aerobic expression of the *fixRnifA*

Corresponding author: H. M. Fischer,
E-mail: hfischer@micro.biol.ethz.ch

Present address of B.Thöny: Abteilung für Klinische Chemie, Kinderspital, Universität Zürich, CH-8032 Zürich, Switzerland.

operon, a random Tn*5*-233 mutagenesis was performed with a *B. japonicum* strain containing a chromosomally integrated *nifA´-´lacZ* fusion as a reporter followed by a screening for mutants with decreased expression of β-galactosidase activity. This strategy led to the identification of a novel *B. japonicum* DNA region (*orf74*) whose mutation indeed affected symbiotic nitrogen fixation but also free-living growth. It turned out, however, that the ORF74 protein is apparently not involved in control of *nifA* expression. Here we report the genetic analysis of the *orf74* DNA region and provide evidence that homologous DNA regions are present in other gram-negative and gram-positive bacteria and even in eukaryotes.

RESULTS

Tn*5*-233 mutagenesis.

A random transposon mutagenesis with the *B. japonicum* strain 7565A2, bearing a chromosomally integrated *nifA´-´lacZ* fusion, was performed and exconjugants with reduced or lacking β-galactosidase activity were screened (Thöny 1989). Among these colonies we expected to find mutants affected in the gene(s) encoding the postulated binding protein involved in the basal expression of the *fixRnifA* operon. About 8,000 exconjugants were tested for their Lac phenotype. Sixty-five of them showed slightly blue and six absolutely white colonies. These were chosen for purification and further analysis. The presence in the chromosome of only one copy of the transposon Tn*5*-233 was ascertained by Southern blot analysis. The Tn*5*-233 insertions in the six white colonies were all located in the integrated *nifA´-´lacZ* fusion. To further substantiate the reduced expression of the integrated *nifA´-´lacZ* fusion in the remaining 65 mutants, a plasmid-borne *fixR´-´lacZ* fusion was introduced into these Tn*5*-233 derivatives and 21 of them still showed a clear reduction in β-galactosidase activity. In a plant infection test with soybean, 10 of these 21 transposon mutants were Fix$^-$, four were Fix$^-$ reduced (5 to 20% activity of the wild type) and seven were Fix$^+$. Among the 10 Fix$^-$ mutants, strain 74 showed a similar nodulation phenotype as observed with a *nifA* mutant (Fischer et al. 1986; Studer et al. 1987). In a gel retardation assay, complex formation between *fixR*-upstream DNA and crude extracts of *B. japonicum* mutant 74 was identical as with wild-type crude extracts, thus ruling out that mutant 74 is affected in the gene encoding the unknown binding protein of the *fixRnifA* promoter region (Thöny 1989). Nevertheless, because of the interesting symbiotic phenotype, mutant 74 was chosen for further characterization.

Genetic characterization of the DNA region affected in mutant strain 74.

A restriction map of the mutated region was established based on Southern blot hybridization of restricted genomic

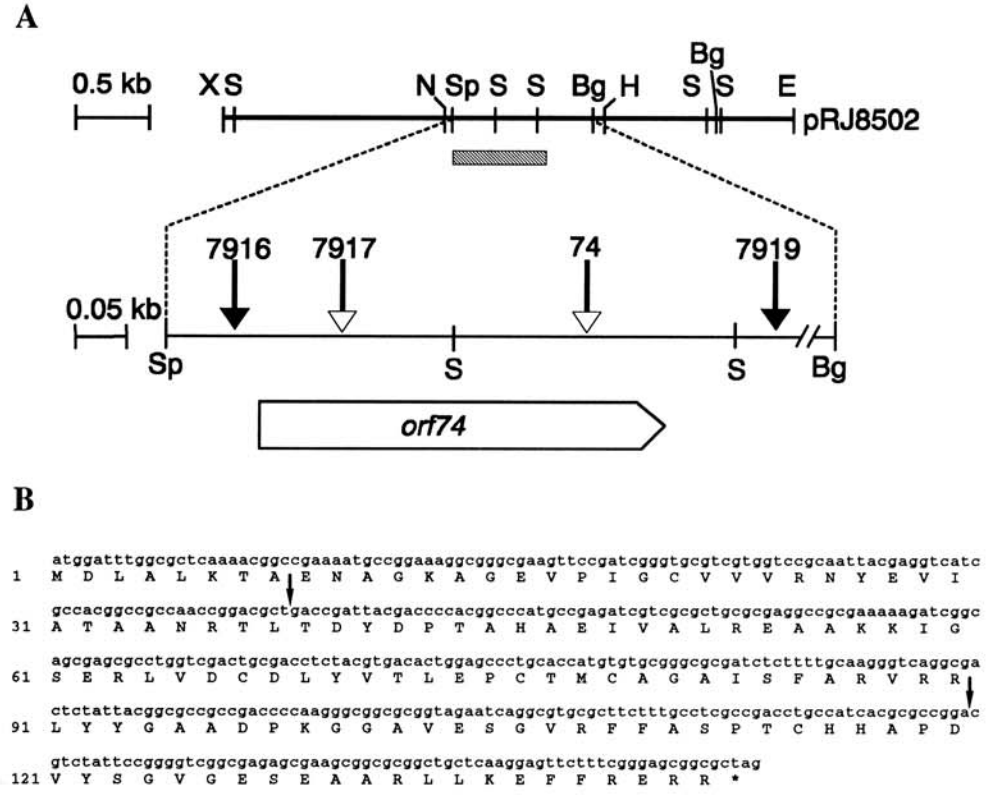

Fig. 1. Restriction map, nucleotide sequence, and deduced amino acid sequence of *Bradyrhizobium japonicum orf74*. **A** shows the restriction map of *orf74* DNA region cloned in plasmid pRJ8502. The hatched bar represents the sequenced DNA region. Open arrowheads indicate the location of Tn*5* and Tn*5*-233 insertions (Fix$^-$), whereas closed arrowheads refer to mutants with wild-type phenotype. Only relevant restriction sites are shown: Bg = *Bgl*II, E = *Eco*RI, H = *Hin*dIII, N = *Nru*I, S = *Sal*I, Sp = *Sph*I, and X = *Xho*I. **B** shows the nucleotide sequence of *orf74* (GenBank accession number: L34743) and the deduced amino acid sequence. Arrows indicate the position of Tn*5*-233 in mutant 74 (closed arrowhead) and Tn*5* in mutant 7917 (open arrowhead). Numbers refer to amino acid positions in the ORF74 protein.

DNA of *B. japonicum* mutant 74 using a Tn*5*-233-internal *Eco*RI–*Xho*I fragment as a probe. Then a 7.35-kb *Sal*I fragment containing the Tn*5*-233 insertion was cloned (Fig. 1A). Subsequently, the corresponding wild-type DNA region was cloned as a 4-kb *Eco*RI–*Xho*I fragment in vector pUC18 using the Tn*5*-233-flanking *B. japonicum* DNA regions of the previously cloned 7.35-kb *Sal*I fragment as a hybridization probe. The physical map of the resulting plasmid pRJ8502 is shown in Figure 1A. DNA sequence analysis of a 700-bp fragment of pRJ8502 (Fig. 1A and B) revealed the presence of an open reading frame termed *orf74* which showed a codon usage typical of the group III *B. japonicum* genes (Ramseier and Göttfert 1991).

The *orf74* region was further characterized by site-directed mutagenesis with transposon Tn*5*. Two insertions resulted in mutant strains (7916 and 7919) with wild-type Nod and Fix phenotypes, whereas a Tn*5* insertion located within *orf74* led to a mutant strain (7917) whose phenotypic properties were identical to those of the original mutant 74 (data not shown; see Fig. 1A, and below). These results support the notion that mutation of *orf74* is the cause for the phenotype of mutant 74 and that the relevant functional region is probably limited to *orf74* itself.

Inspection of a 250-bp DNA region upstream of *orf74* did not uncover any obvious homology to –35/–10 or σ^{54}-dependent –24/–12 canonical promoters (Lisser and Margalit 1993; Kustu et al. 1989; Thöny and Hennecke 1989). Furthermore, no evident similarity to DNA sequence motifs present in the promoter region of *B. japonicum* genes known to be constitutively expressed (unpublished results from our laboratory) could be detected upstream of *orf74*.

To analyze transcription of *orf74*, Northern slot blot hybridization experiments were performed (Fig. 2). A radioactively labeled DNA probe harboring *orf74* was hybridized to RNA isolated both from aerobically grown *B. japonicum* wild-type cells and from wild-type bacteroids. As a control, the same RNA preparations were also hybridized in parallel to a DNA probe harboring the constitutively expressed *B. japonicum sra* gene (Ebeling et al. 1991). As shown in Figure 2, expression of *orf74* was detected under both free-living (aerobic) and symbiotic conditions. The ratio between expression under free-living and symbiotic conditions resembled that of *sra,* but the hybridization signals obtained with the *orf74* probe were approximately 100-fold weaker than those obtained with the *sra* probe. We also attempted to demonstrate expression of *orf74* by means of an in-frame translational *lacZ* fusion to guanine 193 thereby fusing the ORF74 protein at Leu 64 to the ninth amino acid of the LacZ protein. The resulting *orf74′-′*lacZ fusion was cointegrated at the homologous locus into the chromosome of the *B. japonicum* wild-type strain 110*spc*4. However, no β-galactosidase activity expressed from this fusion could be measured in the resulting *B. japonicum* strain 110-97 grown aerobically or anaerobically. Moreover, modification of the β-galactosidase assay to increase the sensitivity of the test did not result in the detection of β-galactosidase activity. These results indicated that under the conditions tested *orf74* of *B. japonicum* is expressed only at a low level or that the stability of the *orf74* transcript or that of the ORF74′-′LacZ fusion protein is extremely weak.

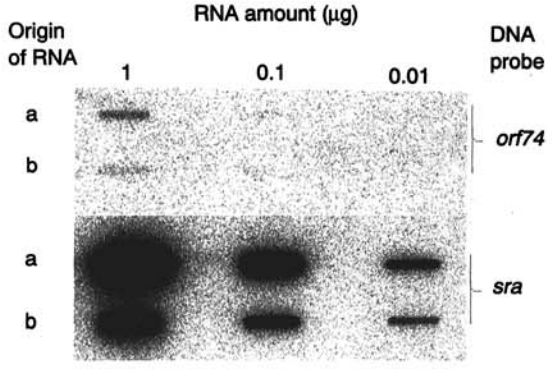

Fig. 2. Autoradiographs of RNA slot blot hybridizations. RNA was isolated from free-living, aerobically grown *Bradyrhizobium japonicum* wild-type cells (a) and from wild-type bacteroids of 21-days old soybean nodules (b). The following DNA fragments were radioactively labeled and used as hybridization probes: *(orf74)*, the 1.2-kb *Hin*dIII–*Bam*HI fragment from pRJ1076 harboring *orf74*; (*sra*), the 0.9-kb *Eco*RI–*Hin*dIII fragment of pRJ2043 bearing the *B. japonicum sra* gene. Hybridized filters were scanned with a Phosphorimager (Molecular Dynamics). Quantification of the data was performed by using the program ImageQuant, version 3.2 (Molecular Dynamics).

Characteristics of the ORF74 protein.

Orf74 encodes a protein of 142 amino acids as deduced from the nucleotide sequence shown in Figure 1B. Its predicted molecular mass is 15.4 kDa and the theoretical isoelectric point pI is 6.74. No transmembrane helices were predicted using computer analysis. Database searches (program TFASTA of the UWGCG sequence analysis software package release 7.3; EMBL database release 41.0) revealed a significant homology of the *B. japonicum* ORF74 protein to proteins present in *Bacillus subtilis* (ORF17; 45.7% identity; Struck et al. 1990), *Escherichia coli* (ORF178; 44.6% identity; Poulsen et al. 1992), *Arabidopsis thaliana* (GenBank accession number: X54385; 39.6% identity; R. Perrey et al., unpublished) and *Lupinus polyphyllus* (GenBank accession number: Z17610; 32.2% identity; M. Raynal et al., unpublished) (Fig. 3). The homology is most pronounced in the region corresponding to amino acids 69 to 82 of *B. japonicum* ORF74. In fact, this sequence motif of 14 amino acids is identical in 13 amino acids of the aligned prokaryotic sequences, including two strictly conserved cysteines at positions 76 and 79. Both cysteine residues are also present in the *L. polyphyllus* sequence, whereas only the first cysteine is conserved in the *A. thaliana* sequence. Twelve proximal amino acids of this motif are partially conserved in deoxycytidylate deaminase from bacteriophage T2 (Struck et al. 1990; Maley et al. 1983) (see Discussion). Thus it appears as if ORF74-homologous proteins are conserved in both prokaryotes and eukaryotes.

Phenotypic characterization of the *B. japonicum* Tn*5*-233 mutant strain 74.

In free-living cultures the *B. japonicum* mutant 74 shows a significantly reduced growth rate as compared with the wild type. The slow growth is observed in rich and minimal medium both under aerobic and anaerobic growth conditions. In PSY medium it is characterized by a prolonged lag-phase of approximately 40 h and a generation time of approximately 24 h during exponential phase, which is about two times longer than that of the wild type (Fig. 4).

In symbiosis the *B. japonicum* mutant 74 shows a Nod$^{+/-}$ Fix^{-} phenotype similar to the symbiotic phenotype of a *B. japonicum nifA* mutant (Fischer et al. 1986). It induces tiny, white, necrotic bumps (Fig. 5A) that are widely dispersed over the roots of 21-day-old soybean plants. These nodules are characterized by empty, fused bacteroid vesicles and by the accumulation of starch. They contain very few, severely degraded bacteroids (Fig. 5B). The residual nitrogen-fixation activity of *B. japonicum* mutant 74 is less than 1% when compared with the wild type, as inferred from an acetylene reduction assay.

Given the reduced growth of mutant 74 it was important to reevaluate the effects of the Tn*5*-233 insertion in mutant 74 on the expression of the *fixRnifA* operon under conditions which allowed a direct comparison between the wild type and mutant 74. To do so, plasmid-borne *lacZ* fusions to *fixR* (pRJ7213) and *nifA* (pRJ7557) were introduced into the wild type and mutant 74. In parallel, a *nifD'-'lacZ* fusion on plasmid pRJ1025 was also conjugated into the same backgrounds. When cultures of these different wild-type and mutant 74 derivatives harboring the aforementioned plasmid-borne *lacZ* fusions were grown aerobically or anaerobically to comparable optical densities no significant differences in β-galactosidase activities derived from any of the *lacZ* fusion plasmids were detected (data not shown). Thus, the mutation in *B. japonicum* strain 74 neither interferes with *nifA* expression nor with NifA activity. The apparent decrease of *fixRnifA* expression in mutant 74 observed during the initial screening procedure must have been a consequence of the reduced growth of this mutant.

Homologous and heterologous complementation experiments.

***B. japonicum* mutant 74.** In view of the pronounced similarity between the *B. japonicum* ORF74 protein and the *B. subtilis* ORF17 protein it was of interest to investigate whether it was possible to functionally complement the *B. japonicum* mutant 74 with *orf17* of *B. subtilis.* Therefore *orf17* of *B. subtilis* was cloned on plasmid pRJ1084, allowing *orf17* to be constitutively transcribed from the *aphII* promoter present on the pRK290 derivative pPG5B (Göttfert et al. 1992; see also Materials and Methods). Plasmid pRJ1084 was then conjugated into *B. japonicum* mutant 74 and tested for its ability to complement the free-living and symbiotic phenotypes of mutant 74. Plasmid pRJ1080, which contains *B. japonicum orf74*, served as the positive (homologous) control in this experiment. In parallel, both *B. subtilis orf17* and *B. japonicum orf74* were also integrated as single copy genes into the chromosome of *B. japonicum* mutant 74 resulting in strains 74-01 and 74-98, respectively (see Materials and Methods). These constructs enabled us to investigate the complementation ability of *orf17* under conditions where no selective pressure for plasmid maintenance can be applied (e.g., symbiosis). In the course of constructing *B. japonicum* strain 74-01 it became evident that a high-copy plasmid containing the *aphII::orf17* fusion is lethal to the *E. coli* DH5α cloning host. Therefore, the *B. japonicum fixR* promoter, which is only very weakly expressed in *E. coli,* was chosen to drive transcription of *B. subtilis orf17* (pRJ1101). In *B. japonicum* 74-01 which contains pRJ1101 chromosomally integrated, *orf17* is transcribed at a basal level under aerobic

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Bj ORF74   1  MDLA----------------------LKTAENAGKAGEV-PIGCVVVRNYEVIATAANRT 37
Bs ORF17      MTQ---------------DELYMKEAIKEAKKAEEKGEV-PIGAVLVINGEIIARAHNLR
Ec ORF178     MRRAFITGVFFLSEVEFSHEYWMRHALTLAKRAWDEREV-PVGAVLVHNNRVIGEGWNRP
At ORF153                          FLTQAVEEAYKGVDCGDGGPFGAVIVHNNVVVASCHNMV
Lp ORF96      MESAIKQANLALYALEV----------------------PVGCVIVEDGKVIASGRNRT
              x                              x  x    xx + + + + x   x    +

Bj ORF74   38 LTDYDPTAHAEIVAL-------REAAKKIGSER--LVDCDLYVTLEPCTMCAGAISFARV 88
Bs ORF17      ETEQRSIAHAEMLVI-------DEACKALGTWR--LEGATLYVTLEPCPMCAGAVVLSRV
Ec ORF178     IGRHDPTAHAEIMAL-------RQGGLVMQNYR--LIDATLYVTLEPCVMCAGAMIHSRI
At ORF153     LKYTDPTAHAEVTAI-------REACKKLNKIE--LSECEIYASCEPCPMSFGAIHLSRL
Lp ORF96      NETRNATRHAEMEAIDVLLEQWQKNGLSMTEIAEIFSKCSLYVTCEPCIMCASALSI---
                     x+++                       x  x    x+xxx+++ +xxx+    x

Bj ORF74   89 RRLYYGAADPKGGAVES---GVRFFASPTCHHAPDVYSGVGESEAARLLKEFFRERR*   142
Bs ORF17      EKVVFGAFDPKGGCSGT---LMNLLQEERFNHQAEVVSGVLEEECGGMLSAFFRELRKKK
Ec ORF178     GRVVFGARDAKTGAAGS---LMDVLHHPGMNHRVEITEGILADECAALLSDFFRMRRQEI
At ORF153     KRLVYGAKAEAAIAIGFDDFIADALRGPRLYQKSSLEIKKADGNGAAIAEQVFQKRKEKF
Lp ORF96      --LV*
                   xx x x x                  x      x    x    x  xxx  x

Bj ORF74
Bs ORF17      KAARKNLS--E*
Ec ORF178     KAQKKAQSSTD*
At ORF153     RLY*
Lp ORF96
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Fig. 3. Alignment of the amino acid sequences of prokaryotic and eukaryotic homologs of *Bradyrhizobium japonicum* ORF74. Bj ORF74 = *B. japonicum* ORF74; Bs ORF17 = *B. subtilis* ORF17 (Struck et al. 1990); Ec ORF178 = *E. coli* ORF178 (Poulsen et al. 1992); At ORF153 = *A. thaliana* amino acid sequence translated from nucleotide 2 to 460 of clone YAP044T (GenBank accession number: X54385; R. Perrey et al., unpublished); the N-terminus of ORF153 is not encoded on this clone; Lp ORF96 = *L. polyphyllus* amino acid sequence translated from nucleotide 2 to 289 of plasmid pPLZ20 (GenBank accession number: Z17610; M. Raynal et al., unpublished). Identical amino acids in prokaryotic proteins are marked with ×; identical amino acids in prokaryotic and eukaryotic proteins are marked with +. The symbol * indicates the C-termini of the aligned ORFs. Numbers refer to amino acid positions in ORF74. The alignment was done using the program CLUSTAL of the UWGCG sequence analysis software package release 7.3.

conditions and is induced approximately fivefold under microaerobic or symbiotic conditions due to the previously described autoregulation of the *B. japonicum fixRnifA* operon (Thöny et al. 1989).

The results of the heterologous complementation experiments with *B. subtilis orf17* as well as those of the controls with the homologous *B. japonicum orf74* are summarized in Table 1. The free-living and symbiotic defects of mutant 74 were complemented by *orf74* independently of its genomic location. By contrast, (partial) heterologous complementation with *B. subtilis orf17* was obtained only when *orf17* was present on a plasmid (pRJ1084) and expressed from the *aphII* promoter. In this case, free-living growth of the complemented mutant 74 was comparable to that of the wild type, whereas nitrogenase activity was restored to only approximately 50% although nodulation was normal. On the other hand, neither the free-living nor the symbiotic defects could be complemented by chromosomal integration of the *fixR::orf17* fusion in strain 74-01. In addition to the slow growth, this strain showed a massive slime production when grown in liquid culture, making it impossible to quantify the growth rate by optical density measurements.

In the complementation experiments it was noticed that the plasmid-bearing strains *B. japonicum* 74/pRJ1080 and 74/pRJ1084 induced two types of nodules which differed in size. The majority were big, whereas some smaller nodules were also present. It was interesting to check whether the size of the nodules correlated with the presence or absence of the respective plasmids. For this purpose, bacteroids of small and large nodules induced by strains 74/pRJ1080 and 74/pRJ1084 were reisolated and assayed for the presence or absence of pRJ1080 and pRJ1084, respectively. Indeed, more than 50% of the bacteroids isolated from big nodules had retained the antibiotic resistance marker despite the absence of selective pressure during the plant infection test. By contrast, more than 90% of the bacteroids isolated from tiny nodules had lost their plasmids.

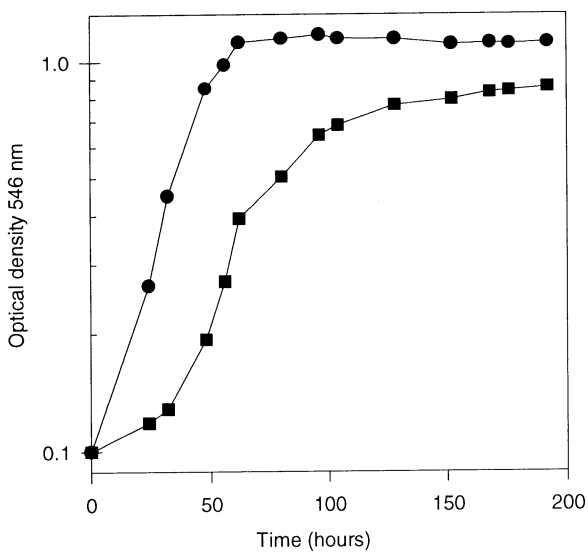

Fig. 4. Growth of *Bradyrhizobium japonicum* wild type (circles) and mutant 74 (squares) in PSY medium under aerobic conditions at 28°C. The curves are based on mean values from four parallel cultures. The size of the standard deviations were smaller than the size of the symbols.

***E. coli* mutant NWL37.** A mutation in the *E. coli orf178* renders the respective mutant strain NWL37 resistant to the cell killing function mediated by the expression of proteins of the *gef* gene family (Poulsen et al. 1992; see Discussion). To explore the functional homology between *B. japonicum* ORF74 and *E. coli* ORF178 we introduced in separate experiments plasmid pRJ1076 and pRJ1118 into the *E. coli* mutant NWL37 containing pSM970 which allows for IPTG-inducible expression of *gef* (Poulsen et al. 1992). Plasmid pRJ1076 carries *orf74* expressed from its genuine promoter, whereas plasmid pRJ1118 harbors *orf74* under the control of the constitutive *aphII* promoter. As a homologous control, plasmid pLKP166 (Poulsen et al. 1992) which expresses the wild-type *orf178* was introduced into the same *E. coli* background (for further details see Materials and Methods). The induction of *gef* on pSM970 by IPTG caused *E. coli* mutant NWL37 cells to die only when pLKP166 was present in the cells; however, the *B. japonicum* ORF74 protein was unable to promote cell death independently of its mode of expression (results not shown).

In conclusion, it was shown on one hand that the *B. japonicum* mutant 74 phenotypes can be complemented by the homologous *B. japonicum orf74* and also by the *B. subtilis orf17,* provided the latter is located on a plasmid and expressed from the constitutive *aphII* promoter. On the other hand, the homology of the *B. japonicum* ORF74 protein with the *E. coli* ORF178 protein was not reflected at the functional level since an involvement of ORF74 in the *E. coli gef*-mediated cell-killing mechanism could not be demonstrated.

DISCUSSION

The *B. japonicum* DNA region presented in this paper harbors an open reading frame encoding the ORF74 protein, which shows approximatively 45% identity to ORF17 of *B. subtilis* and ORF178 of *E. coli.* The homology is limited to the protein-encoding regions because no obvious DNA sequence similarities were found in the upstream or downstream regions of the respective *orfs.* The function of the *B. subtilis* ORF17 is not known and no mutational data is available so far. The relevance of the homology of a 16-amino-acid peptide of ORF17 (positions 72 to 87) to deoxycytidylate deaminase from bacteriophage T2 (Struck et al. 1990; Maley et al. 1983) remains uncertain because only 10 and eight of these amino acids are conserved in *E. coli* ORF178 and *B. japonicum* ORF74, respectively. The similarity between *B. japonicum* ORF74 and *B. subtilis* ORF17 is further substantiated by the results of the heterologous complementation experiments with mutant 74. The free-living and symbiotic defects of mutant 74 could be corrected at least partially provided that a minimal critical level of *orf17* expression is brought about. However, no complementation was obtained when only one copy of *orf17* was present and when its transcription was controlled by the *B. japonicum fixR* promoter. Although this promoter is active at a basal level under aerobic growth conditions and induced about fivefold in symbiosis (Thöny et al. 1989) it is probably not able to direct the synthesis of sufficient amounts of ORF17 protein when *orf17* is present as a single-copy gene in *B. japonicum* 74-01. On the other hand, extreme overexpression of *orf17* is probably deleterious to *E. coli* cells, as it was not possible to clone the

aphII::orf17 fusion on a high-copy vector. It must be concluded, therefore, that the level of expression of ORF17, and by analogy ORF74, in the cell is extremely delicate and that quantitative and/or qualitative disturbances have dramatic consequences on the general metabolism and, consequently, on the symbiotic effectiveness of *B. japonicum* cells.

In the case of the *E. coli orf178* a missense mutation has been characterized (Poulsen et al. 1992). The resulting *E. coli* mutant strain was transiently resistant to the cell-killing function encoded by the *gef* gene family. This family includes four members that are located on conjugative plasmids of different incompatibility groups (e.g., *hok, flmA, pndA,* and *srnB*; Gerdes et al. 1990) and two chromosomally encoded members (e.g., *relF* and *gef*; Bech et al. 1985; Poulsen et al. 1991). While the first are involved in plasmid stabilization through postsegregational killing of plasmid-free cells, the role of the latter in the cell-killing mechanism is still unknown. On the basis of the Gef-resistant phenotype of the *E. coli orf178* strain it was speculated that the ORF178 protein may interact directly with the membrane-bound Gef protein (Poulsen et al. 1992); however, no direct evidence is available to support this hypothesis. *Rhizobium loti* and cowpea *Rhizobium* ANU240 might possess *gef*-homologous DNA sequences as inferred from hybridization experiments with an *E. coli gef* DNA probe (Poulsen et al. 1989); however, at least in *R. loti* no *orf178*-like DNA region appears to be present (Poulsen et al. 1992). Our results from the heterologous complementation experiments using the *E. coli* mutant NWL37 and the *B. japonicum* ORF74 protein suggest that the *orf74* DNA region of *B. japonicum* is probably not involved in the cell-killing mechanism mediated by the *gef* gene family in *E. coli*. It remains to be elucidated whether a similar cell killing mechanism exists at all in the rhizobia, in which ORF74 might play a role.

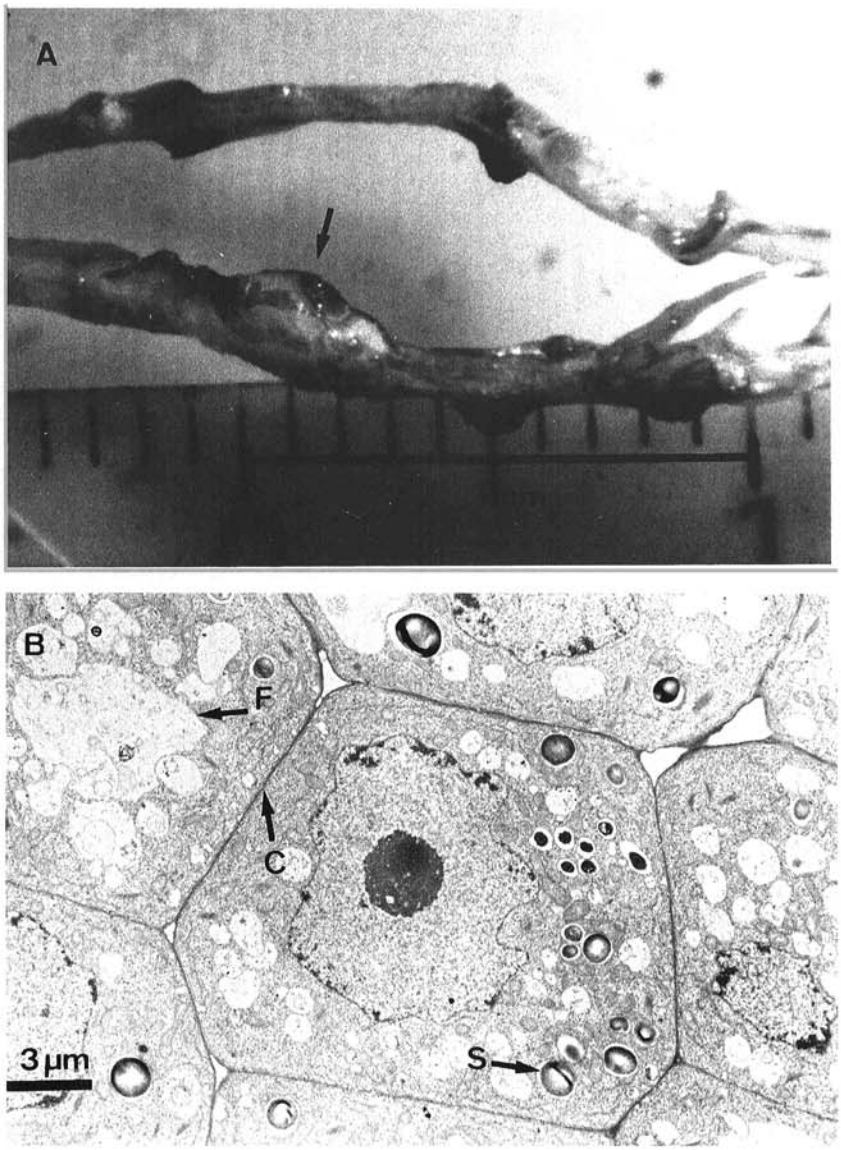

Fig. 5. Symbiotic phenotype of mutant 74. **A**, Macroscopic view of 21-days-old soybean roots infected with *Bradyrhizobium japonicum* mutant 74. The arrow marks a characteristic tiny nodulelike structure (bump). **B**, Electron micrograph showing the ultrastructure of a 21-days-old soybean nodule elicited by *B. japonicum* mutant 74. Abbreviations: C = plant cell wall, F = fused vesicles, S = starch.

Northern slot blot hybridizations demonstrated that *orf74* is expressed under free-living and symbiotic conditions. The level of expression, however, appears to be rather low, which was further substantiated by our failure to detect expression by means of β-galactosidase activity originating from an *orf74′-′lacZ* fusion.

A typical –35/–10 promoter has been shown to be essential for transcription of *B. subtilis orf17* (Struck et al. 1990), but no promoter of this type was found upstream of *E. coli orf178*. It was speculated that the latter is organized in an operon and cotranscribed with *orf190* located upstream of *orf178* (Poulsen et al. 1992). In fact, *orf190* is preceded by a –35/–10 promoter sequence, but its function has not been proven. No obvious promoter element or another open reading frame could be identified by visual inspection of an approximately 200-bp DNA region upstream of *B. japonicum orf74*. The fact that the 1.2-kb *Nru*I–*Hin*dIII fragment spanning *orf74* could complement mutant 74 in *trans* strongly indicates that this DNA region contains all of the elements required for expression of *orf74*. The Nod$^+$Fix$^+$ phenotype of mutant 7916, which carries a Tn*5* insertion only 19 bp upstream of the putative *orf74* start codon, means that either no functional DNA region is affected by this insertion or that an outreading promoter activity from the transposon can substitute for the hypothetical *orf74* promoter.

Initially, this work aimed at the genetic identification of a regulatory gene postulated to be required for basal expression of the *B. japonicum fixRnifA* operon (see Introduction). The symbiotic phenotype of mutant 74 indeed resembled that of a previously described *nifA* mutant (Fischer et al. 1986; Studer et al. 1987), suggesting that the mutated gene might be involved in either *nifA* expression or NifA activity. However, our results from the expression studies with the *fixR′-*, *nifA′-* and *nifD′-′lacZ* fusions in the mutant 74 background now argue against this idea. Alternatively, it could have been possible that *orf74* whose product is essential for a functional symbiosis represents a target gene for control by NifA. The existence of such genes had been suggested on the basis of the analysis of two-dimensional protein gels with extracts from a *nifA* mutant (Fischer et al. 1986). However, the absence of regulatory elements typical for NifA-controlled genes upstream of *orf74* and the expression of *orf74* under aerobic growth conditions in which NifA is inactive speak against a NifA-dependent expression of *orf74*. Thus, it is unlikely that there is any link between *nifA* and *orf74* at the genetic level. Furthermore, the free-living phenotype (slower growth) of mutant 74 implies that the observed symbiotic phenotypes may be interpreted as a consequence of a disturbance in a more general function of the bacterial metabolism, not necessarily restricted to bacteroid development and/or symbiotic nitrogen fixation. In fact, the wide distribution of ORF74-homologous proteins in nitrogen non-fixing bacteria and in eukaryotes supports this argument. Examples of *B. japonicum* genes encoding such functions include the *B. japonicum glyA* gene involved in glycine biosynthesis (Rossbach and Hennecke 1991), genes involved in bacterial respiration (*fixNOQP*; Hennecke, 1993) and in the biogenesis of cytochromes (*cycVWX*; Thöny-Meyer et al. 1994) and heme (*hemB*; Chauhan and O'Brian 1993), genes for C_4-dicarboxylate transport (Humbeck and Werner 1987; Humbeck and Werner 1989), or the *groESL* chaperonin genes (Fischer et al. 1993; unpublished results from our laboratory). Thus, at the present time, we conclude that ORF74 exerts a basic physiological function concerned with growth in general and consequently also with symbiosis and, therefore, it seems not appropriate to call *orf74* a true symbiotic gene.

MATERIALS AND METHODS

Bacterial strains, vectors, plasmids, media, and growth conditions.

The strains, vectors, and plasmids used in this work are listed in Table 2. *B. japonicum* 110*spc*4 is referred to as wild type in this paper. *B. japonicum* strains were grown aerobically in PSY medium (Regensburger and Hennecke 1983) at 28°C with vigorous shaking, and anaerobically in YEM medium containing 10 mM KNO_3 (Daniel and Appleby 1972) at 28°C in tightly closed serum bottles flushed with argon. Antibiotics for selection were used as follows (μg/ml): spectinomycin 100, kanamycin 100, gentamycin 100, tetracycline 50. *E. coli* strains were grown in LB medium (Miller, 1972) at 37°C. Antibiotics in cultures of *E. coli* were used at the following concentrations (μg/ml): ampicillin 200, kanamycin 50, and tetracycline 10.

DNA and RNA manipulations.

Cloning, restriction mapping, Southern blotting, and hybridizations were done according to standard procedures (Sambrook et al. 1989). RNA was isolated from aerobically grown *B. japonicum* cells and from bacteroids as described by Gubler and Hennecke (1988). Northern slot blot hybridizations were performed as published earlier (Weidenhaupt et al.

Table 1. Complementation analysis of *Bradyrhizobium japonicum* mutant 74

		Phenotype		
			Symbiotic	
***B. japonicum* strain**	**Relevant characteristics**	**Free-living[a]**	**Fix[b]**	**Nod[c]**
110*spc*4	Wild type	wt	99.8 ± 29.3	+
Mutant 74	Tn*5*-233 in *orf74*	mut	0	+/–
74/pRJ1080	Mutant 74 with plasmid-borne *orf74*	wt	66.1 ± 19.8	+
74-98	Mutant 74 with chromosomally integrated *orf74*	wt	120.1 ± 34.7	+
74/pRJ1084	Mutant 74 with plasmid-borne p_{tuphII}::orf17	wt	45.9 ± 20.8	+
74-01	Mutant 74 with chromosomally integrated p_{fixR}::*orf17*	mut, slimy	0	+/–

[a] wt = Normal growth (doubling time 14 h), mut = slow growth (doubling time 24 h) in PSY medium at 28°C.
[b] Specific nitrogenase activity in μmol C_2H_2 produced x h^{-1} x g^{-1} nodule dry weight measured from six 21-day-old soybean plants.
[c] + = Wild-type nodulation, +/– = mutant 74-typical nodulation (for further details see Results).

1993). DNA hybridization probes were radioactively labeled by using the nick-translation technique described by Sambrook et al. (1989). DNA sequencing was performed according to the chain termination method of Sanger et al. (1977) using bacteriophage M13 subclones.

Construction of plasmids and *B. japonicum* mutant strains.

Both plasmids pRJ1080 and pRJ1084 are based on derivatives of the broad host range vector pRK290. A 1.2-kb *Hin*dIII-*Nru*I fragment spanning *B. japonicum orf74* (Fig. 1A) was cloned (after *Bam*HI linker addition to the *Nru*I site) into the *Hin*dIII-and *Bam*HI-digested vector pRKPol2, resulting in plasmid pRJ1080. pRJ1084 harbors a 0.7-kb *Bgl*II-*Pvu*II fragment of pSPORF17 (kindly provided by R. Kretschmer-Kazemi Far) containing the *B. subtilis orf17*. Prior to ligation, a *Bam*HI linker was added to the *Pvu*II site of this fragment, which was subsequently cloned into the vector pPG5B digested with *Bam*HI. In this construct, *B. subtilis orf17* is transcribed from the *aphII* gene present on pPG5B. Both plasmids were transformed into *E. coli* S17-1 and conjugated into *B. japonicum* mutant 74.

Chromosomal integration of *B. japonicum orf74* and *B. subtilis orf17* into the *B. japonicum* mutant 74 background was performed using the vector pRJ1035 which allows site-directed genomic integration by homologous recombination via the nonessential repetitive sequences RSα and RSβ present on the *B. japonicum* chromosome (Acuña et al. 1987). In this experiment, tetracycline was used to select for single crossing-over events. Plasmid pRJ1098 contains a 1.2-kb *Eco*RI–*Nru*I fragment (see Fig. 1A) spanning the entire *B. japonicum orf74* cloned into the *Eco*RI and *Sma*I cloning sites of pRJ1035. Plasmid pRJ1101 originates from a previously constructed plasmid, pRJ7276. A 5-kb *Bam*HI fragment spanning the *lacZ* portion of a *fixR´-´lacZ* fusion present on pRJ7276 was replaced by a 0.7-kb *Bgl*II–*Bam*HI fragment

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference
B. japonicum		
110*spc*4	Spc^r	Regensburger and Hennecke 1983
7565A2	Spc^r, Km^r, chromosomally integrated *nifA´-´lacZ* fusion	Thöny et al. 1987
74	Spc^r, Km^r, Gm^r	This work
7916	Km^r	This work
7917	Km^r	This work
7919	Km^r	This work
110-97	Spc^r, Tc^r, chromosomally integrated *orf74´-´lacZ* fusion	This work
74-98	Spc^r, Km^r, Gm^r, chromosomally integrated wild-type *orf74*	This work
74-01	Spc^r, Km^r, Gm^r, chromosomally integrated *B. subtilis orf17*	This work
E. coli		
DH5α	*supE*44, Δ*lacU*169 (Φ80*lacZ*ΔM15) *hsdR*17, *recA*1, *endA*1, *relA*1	BRL, Gaithersburg, MD, USA
JM101	Δ(*lac-proAB*), *thi, supE,* F´(*traD*36, *proAB*$^+$, *lacI*^{q} Z ΔM15)	Messing 1983
S17-1	*hsdR*, RP4-2, *kan*::Tn*5*, *tet*::Mu, chromosomally integrated	Simon et al. 1983
MM294A	*pro*-82, *thi*-1, *endA*1, *hsdR*17, *supE*44	De Vos et al. 1986
NWL37	*gef-lacZ*, Δ*relF*, Cm^r, Km^r, Gef^r	Poulsen et al. 1992
Plasmids		
M13 mp18/19	Sequencing vectors	Norrander et al. 1983
pBluescript SK+	Ap^r	Stratagene, La Jolla, CA, USA
pUC4-KIXX	Km^r	Pharmacia, Uppsala, Sweden
pSP72	Ap^r	Promega, Madison, WI, USA
pSUP202	Tc^r	Simon et al. 1983
pNM482	Ap^r	Minton 1984
pRKPol2	Tc^r, (pRK290), multiple cloning site of pBluescript SK+	Göttfert et al. 1992
pPG5B	Tc^r, (pRK290), containing *aphII* on a 1.65-kb *Hin*dIII fragment from pUC4-KIXX	P. Grob, unpublished
pRJ1035	Tc^r, Km^r, (pRK202) site-directed integration vector	Acuña et al. 1987
pRK2013	Km^r, *tra*$^+$, (ColE1)	Figurski and Helinski 1979
pRK607	Km^r, Gm^r, Str^r, Spc^r, (pRK2013), Ω::Tn5-233	De Vos et al. 1986
pSPORF17	Ap^r, (pSP72), *B. subtilis orf17* on a 0.65-kb *Eco*RI-*Sal*I fragment	R. Kretschmer-Kazemi Far, unpublished
pSM970	Tc^r, IPTG-inducible *gef*	Poulsen et al. 1992
pLKP166	Ap^r, wild-type *orf178*	Poulsen et al. 1992
pRJ1025	Tc^r, (pRK290), *nifD´-´lacZ* fusion	Alvarez-Morales et al. 1986
pRJ1076	Ap^r, (pUC18), *B. japonicum orf74* on a 1.2-kb *Hin*dIII-*Nru*I fragment	This work
pRJ1080	Tc^r, (pRKPol2), *B. japonicum orf74* on a 1.2-kb *Hin*dIII-*Nru*I fragment	This work
pRJ1084	Tc^r, (pPG5B), *B. subtilis orf17* on a 0.7-kb *Bgl*II-*Pvu*II fragment	This work
pRJ1094	Ap^r, (pNM482), *B. japonicum orf74* on a 0.35-kb *Nru*I-*Sal*I fragment	This work
pRJ1097	Tc^r, (pSUP202), *B. japonicum orf74´-´lacZ* on a 5-kb *Eco*RI-*Stu*I fragment	This work
pRJ1098	Tc^r, (pRJ1035), *B. japonicum orf74* on a 1.2-kb *Eco*RI-*Nru*I fragment	This work
pRJ1101	Tc^r, (pRJ7276), *B. subtilis orf17* on a 0.68-kb *Bgl*II-*Bam*HI fragment	This work
pRJ1118	Ap^r, (pUC4-KIXX) p_{aphII}::*orf74* on a 1.2-kb *Nru*I-*Hin*dIII fragment	This work
pRJ2043	Ap^r, (pGEM1) *B. japonicum sra*	Ebeling et al. 1991
pRJ7213	Tc^r, (pRK290), *fixR´-´lacZ*	Thöny et al. 1987
pRJ7276	Tc^r (pRJ1035), *fixR´-´lacZ*	B. Thöny, unpublished
pRJ7557	Tc^r, (pRK290), *nifA´-´lacZ*	Thöny et al. 1987
pRJ8502	Ap^r, (pUC18), *orf74* on a 4-kb *Eco*RI-*Xho*I fragment	M. Schmid-Appert, unpublished

from pSPORF17 bearing the *B. subtilis orf17*, thereby placing *orf17* under the transcriptional control of the *B. japonicum fixR* promoter. Both plasmids (pRJ1098 and pRJ1101) were transformed into *E. coli* S17-1 cells and conjugated into *B. japonicum* mutant 74. Chromosomal integrations were selected on tetracycline-containing PSY plates. Total DNA from candidates putatively containing the plasmids cointegrated in the chromosome was analyzed by Southern blot hybridization. The resulting strains *B. japonicum* 74-98 and *B. japonicum* 74-01 contain plasmids pRJ1098 and pRJ1101, respectively, cointegrated at the RSβ3 locus in the *B. japonicum* chromosome.

The translational *orf74′-′lacZ* fusion on plasmid pRJ1094 was constructed by cloning the 0.35-kb *Nru*I–*Sal*I fragment of *B. japonicum orf74* (see Fig. 1A) into the *Bam*HI–*Sal*I restricted vector pNM482 after addition of a *Bam*HI linker to the *Nru*I end. To integrate the *orf74′-′lacZ* fusion into the chromosome of the wild-type *B. japonicum*, a 5-kb *Eco*RI-*Stu*I fragment of pRJ1094 containing the entire *orf74′-′lacZ* fusion was cloned into the *Eco*RI and *Pst*I digested vector pSUP202 after *Pst*I linker ligation to the *Stu*I site. The resulting plasmid, pRJ1097, was transformed into *E. coli* S17-1 and conjugated into the wild-type *B. japonicum*. The expected genomic structure of the cointegrate-containing strain *B. japonicum* 110-97 was confirmed by Southern blot analysis.

For expression of *orf74* under the control of the *lac* promoter a 1.2-kb *Nru*I–*Hin*dIII fragment harboring *orf74* (see Fig. 1A) was ligated to the *Hin*dIII–*Bam*HI-linearized vector pUC18 after addition of a *Bam*HI linker to the *Nru*I ends. This resulted in plasmid pRJ1076. The same *Nru*I–*Hin*dIII fragment was also ligated to the *Nru*I linearized vector pUC4-KIXX after filling-in the protruding 5′-*Hin*dIII ends with Klenow DNA polymerase. In the resulting plasmid pRJ1118 *orf74* is constitutively expressed from the *aphII* promoter.

Tn*5*-233 mutagenesis.

B. japonicum strain 7565A2 carries a chromosomally integrated *nifA′-′lacZ* fusion and yields dark-blue colonies on PSY plates containing X-Gal (90 μg/ml) after 9 days of incubation at 28°C, due to the aerobic expression of the *fixRnifA* operon. A transposon insertion in the gene(s) encoding a putative activator for the *fixRnifA* expression was expected to yield white or faint blue colonies on X-Gal containing PSY plates. Because of the previous integration of a *nifA′-′lacZ* fusion, *B. japonicum* strain 7565A2 already carried a Tn*5*-derived *aphII* gene in the chromosome and thus a target sequence for the integration of Tn*5* by homologous recombination. Therefore the random transposon mutagenesis was performed with a Tn*5* derivative, Tn*5*-233, which carries the resistance genes for the antibiotics gentamycin/kanamycin and streptomycin/spectinomycin instead of *aphII* (De Vos et al. 1986). The *E. coli* strain MM294A containing pRK607 (kindly provided by E. R. Signer) was used as a donor strain for the mating with *B. japonicum* 7565A2. Matings were performed either on nitrocellulose filters as described (Hahn and Hennecke 1984) or by directly spotting donor and recipient cells on PSY plates (Göttfert et al. 1989). To eliminate spontaneous resistant mutants, selection for exconjugants was done on PSY plates containing both gentamycin (200 μg/ml) and streptomycin (100 μg/ml). Chloramphenicol (10 μg/ml) was used to counterselect against the *E. coli* donor, and X-Gal (90 μg/ml) to monitor the expression of the integrated *nifA′-′lacZ* fusion. After incubation at 28°C for 15 to 20 days, double-resistant (Gm^r, Str^r) colonies arose at a frequency of 2.5×10^{-8}. Approximately 8,000 exconjugants were tested for their Lac phenotype.

Plant infection test and acetylene reduction measurement.

Plant infection tests with soybean (*Glycine max* cv. Williams) were performed as described previously (Hahn and Hennecke 1984). It is known that pRK290-based plasmids of *B. japonicum* are lost during a plant infection test because no antibiotic selective pressure can be applied (Rossbach and Hennecke 1991). Therefore, it was necessary to estimate the ratio of plasmid-containing, reisolated bacteroids in order to correctly interpret results from complementation experiments. To do so, root nodules were sterilized by rinsing in 96% ethanol and afterwards by washing with sterile water (three nodules of two plants were analyzed for each strain). The nodules were crushed and serial dilutions of the bacteroid suspensions were plated in parallel on PSY plates containing or lacking tetracycline. To inhibit growth of fungi, cycloheximide was added at 100 μg/ml. The ratio of plasmid-containing bacteroids was estimated by comparing the numbers of colonies growing in the presence and absence of tetracycline. Symbiotic nitrogen-fixation activity was measured by the acetylene reduction assay (Turner and Gibson 1980).

β-Galactosidase assays.

The expression of *lacZ* fusions was measured as described by Miller (1972). In those cases where the expression was very low, β-galactosidase activity measurements were modified as described by Grob et al. (1993) to increase the sensitivity of the test.

Complementation of *E. coli* mutant strain NWL37.

E. coli NWL37 cells harboring pSM970 were transformed separately with plasmids pRJ1076, pRJ1118, and pLKP166. Transformants were grown at 37°C in liquid cultures of 20-ml LB medium containing appropriate antibiotics until an OD_{550} of about 0.3 was reached. The cultures were then divided into two 10-ml cultures. IPTG (0.5 mM) was added to one subculture for induction of *gef* gene expression. As control, the second subculture of each strain was grown further without addition of IPTG. Subsequent growth of all subcultures was monitored by OD_{550} measurements in intervals of 30 min for 4 h. Gef^s strains were characterized by a retarded growth of the IPTG-induced subculture as compared with the uninduced control, whereas in Gef^r strains growth was not affected by *gef* induction via IPTG addition. Additional controls included *E. coli* NWL37 harboring pSM970 as the only plasmid as well as *E. coli* NWL37 harboring only pRJ1076, pRJ1118, or pLKP166.

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