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Genetic and Physiologic Characterization of a *Bradyrhizobium japonicum* Mutant Defective in Early Bacteroid Development

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We report the identification and cloning of a new symbiotically essential gene from the soybean root nodule bacterium, *Bradyrhizobium japonicum*. The main phenotypic characteristics of mutants with insertions in that gene (e.g., strain 3160) are as follows: 1) mutant 3160 forms numerous tiny, bacteroid-free pseudonodules on soybean and three other legume hosts; 2) the absence of endosymbiotic bacteria is strictly correlated with a selective decrease in the expression of the soybean early nodulin gene ENOD55, whereas ENOD2 and ENOD13 are expressed normally; 3) mutant 3160 does not succeed in bacteroid propagation even in the presence of other *B. japonicum* strains as coinoculants, whereas it is able to restore the full nodulation capability of coinoculant *nodA* and *nodC* mutants; 4) strain 3160 has a leaky folate auxotrophy when grown on minimal medium with nitrate as the sole nitrogen source. The affected gene appears to play an important role in a very early stage of bacteroid and root nodule development.

Additional keywords: gene cloning, nitrogen fixation, root nodule symbiosis, signal exchange, vitamin auxotrophy.

In the relationship between rhizobia and legumes many bacterial genes have been identified that play an important role in establishing an effective, nitrogen-fixing root nodule symbiosis. There are three well-known groups of genes, the *nod*, *nif*, and *fix* genes. Nodulation (*nod*) genes are necessary for the very early steps of plant root infection (for reviews, see Long 1984; Morrison *et al.* 1988; Rolfe and Gresshoff 1988). The *nif* genes are all genes homologous to the essential nitrogen fixation genes of *Klebsiella pneumoniae* (Schroeter) Trevisan, a free-living diazotroph. All other genes that have turned out to be necessary for an effective symbiosis have been called *fix* genes.

The *fix* gene terminology, however, is somewhat misleading and inappropriate in cases in which the corresponding genes determine functions in bacteroid and nodule development. Several rhizobial mutants have been described that were not able to elicit fully developed nodules. For example, mutants of *Rhizobium meliloti* Dangeard disturbed in exopolysaccharide synthesis (Exo^-) caused empty, white nodules on alfalfa (Finan *et al.* 1985; Leigh *et al.* 1987). Similarly, a genetic locus (*ndvA*) for β-(1→2)-glucan synthesis in *R. meliloti* has been shown to be necessary for normal nodule development (Dylan *et al.* 1986; Stanfield *et al.* 1988). Noel *et al.* (1988) reported that a specific nutrient requirement can also become a limiting factor in the development of a normal nodule: they showed that purine auxotrophs of *R. leguminosarum* bv. *phaseoli* Jordan were not able to induce fully developed nodules on beans.

Our laboratory has an interest in identifying new bacterial genes that are essential for effective root nodule symbiosis between *Bradyrhizobium japonicum* (Buchanan) Jordan and soybean (*Glycine max* (L.) Merr.). After random Tn*5*-induced mutagenesis of the *B. japonicum* genome we previously obtained mutants that nodulated soybean but could not fix nitrogen (phenotype Nod^+Fix^-) (Regensburger *et al.* 1986). One of these mutants was of particular interest, as it was obviously disturbed in an early step of symbiosis. This mutant (strain 3160) led to tiny, white nodules on soybean, without any clearly identifiable infection threads and bacteroids. In this report we present a comprehensive genetic and physiologic analysis of this mutant, with the aim of possibly understanding the biochemical basis of its defect.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are shown in Table 1.

Media and bacterial growth conditions. *Escherichia coli* was grown in Luria-Bertani medium (Miller 1972) at 37° C. Rich medium for *B. japonicum* was peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke 1983). Minimal *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid and 2-(*N*-morpholinoethane sulfonic acid) (HM) medium was prepared according to Cole and Elkan (1973) with 1% arabinose as the carbon source. *B. japonicum* was routinely grown at 28–30° C. Antibiotics were added at the following concentrations: ampicillin, 50–100 μg/ml; chloramphenicol, 20 μg/ml (*E. coli*) or 10 μg/ml (*B. japonicum*); kanamycin, 20 μg/ml (*E. coli*) or 100–200 μg/ml (*B. japonicum*); rifampicin, 100 μg/ml; streptomycin, 100–200 μg/ml; spectinomycin 100–200 μg/ml; and tetracycline, 10 μg/ml (*E. coli*) or 100–120 μg/ml (*B. japonicum*).

DNA isolation and manipulation. Total DNA of *B. japonicum* was isolated as described by Hahn and Hennecke (1984). Large-scale plasmid isolation was done by the

cleared lysate method of Itoh *et al.* (1984). Small-scale plasmid isolation was done by alkaline lysis as described by de Bruijn and Lupski (1984). Restriction endonuclease digests, gel electrophoresis, Southern blotting, nick translation, hybridization, and transformation were done according to Maniatis *et al.* (1982).

Marker replacement mutagenesis. Marker replacement mutagenesis was done as described by Hahn and Hennecke (1984), with *E. coli* strain S17-1 as donor and plasmid pSUP202 derivatives for mobilization (Simon *et al.* 1986).

Nodulation and nitrogen fixation assays. Nodulation tests on *G. max* 'Williams' and nitrogen fixation assays (free-living and *in planta*) were performed as described previously (Hahn and Hennecke 1984; Regensburger *et al.* 1986).

Coinoculation experiments. Two different strains of *B. japonicum* were grown for 5 days to $A_{600} = 1.0$, and 2-day-old seedlings of *G. max* were coinoculated with them in a 1:1 ratio. After 21 days the plants were scored for nodulation, and whole-nodule nitrogen fixation activity was determined by the acetylene reduction assay (Turner and Gibson 1980).

Isolation of bacteria from nodules. Nodules were surface-sterilized (1 min in 96% ethanol) and washed four times with sterilized water. Then they were put into an Eppendorf tube containing 1 ml of 0.9% NaCl and crushed with sterile forceps. Dilutions of 10^{-2} and 10^{-4} were plated on PSY agar containing appropriate antibiotics (if necessary) and

Table 1. Bacterial strains and plasmids

	Relevant characteristics[a]	Origin
Bacteria		
Escherichia coli		
RR28	*hsdR hsdM recA pheS12 thi leu pro*	Hennecke *et al.* 1982
HB101	*hsdS20 recA13 thi leu proA2*	Boyer and Roulland-Dussoix 1969
S17-1	*hsdR thi pro*, RP4-2 *tet*::Mu *kan*::Tn*7* integrated in the chromosome	Simon *et al.* 1986
Bradyrhizobium japonicum		
110rif15	Rif^r, referred to as "wild type"	Regensburger and Hennecke 1984
3160[b]	Rif^r, Tn*5*(Km^r Str^r)	Regensburger *et al.* 1986
2325Ω[b]	Rif^r, Ω insertion (Sp^r Str^r)	This work
Δ2330Ω[b]	Rif^r, 0.5-kb deletion plus Ω insertion (Sp^r Str^r)	This work
B3	Sp^r, *nifB*::*aph*(Km^r)	Ebeling *et al.* 1987
168	Sp^r, *nodC*::*aph*(Km^r)	Göttfert *et al.* 1989
335	Sp^r, *nodA*::*aph*(Km^r)	Lamb and Hennecke 1986
61A76	Wild-type isolate	NifTAL[c]
Bradyrhizobium sp. 32H1 (*Crotalaria*)		Nitragin[d]
Bradyrhizobium sp. ANU289 (*Parasponia*)		P. Gresshoff
Rhizobium meliloti 41 (AK631)		A. Kondorosi
R. leguminosarum bv. *phaseoli* 8002		Lamb *et al.* 1982
Rhodopseudomonas palustris 123		DSM[e]
Plasmids		
pBR329	Ap^r Cm^r Tc^r	Covarrubias and Bolivar 1982
pUC18	Ap^r, *lacZ*(α)	Norrander *et al.* 1983
pLAFR1	Tc^r cos *oriT*(RK2)	Friedman *et al.* 1982
pSUP202	Ap^r Cm^r Tc^r *oriT*(RP4)	Simon *et al.* 1986
pSUP202-E	Like pSUP202, minus *Eco*RI site	M. Hahn
pHP45Ω	Ap^r Sp^r Str^r, source of Ω	Frey and Krisch 1985
pL44-8C[f]	Tc^r(pLAFR1), 19.2-kb wild-type *B. japonicum* DNA from "3160 region"	This work
pRJ2300[f]	Ap^r Tc^r(pBR329) Km^r(Tn*5*), 7.5-kb Tn*5*-containing *Eco*RI fragment from 3160	This work
pRJ2301	Ap^r Tc^r(pSUP202) Km^r(Tn*5*), same insert as pRJ2300	This work
pRJ2316[f]	Ap^r(pUC18) Km^r(Tn*5*), 19-kb Tn*5*-containing *Asp*718 fragment from 3160	This work
pRJ2325Ω[f]	Tc^r(pSUP202-E) Sp^r Str^r(Ω), 2.3-kb *Pst*I fragment with Ω insertion in *Eco*RI site	This work
pRJ2330Ω[f]	Tc^r (pSUP202-E) Sp^r Str^r(Ω), 3.6-kb *Bgl*II fragment with Ω insertion replacing a 0.5-kb internal *Eco*RI fragment	This work

[a] Ap = ampicillin; Cm = chloramphenicol; Km = kanamycin; Rif = rifampicin; Sp = spectinomycin; Str = streptomycin; Tc = tetracycline; ^{r} = resistant.
[b] The insertions of these mutants are shown in Figure 3.
[c] Rhizobium Germplasm Resource Center (NifTAL), Paia, Hawaii.
[d] Nitragin Co., Milwaukee, WI.
[e] Deutsche Sammlung für Mikroorganismen, Göttingen, West Germany.
[f] The inserts of these plasmids are shown in Figure 3.

cycloheximide (100 μg/ml) to prevent fungal growth.

Enzymatic assay. Nitrate reductase activity was determined by measuring the consumption of nitrate in the medium. Nitrate was chemically reduced to nitrite with zinc dust, and the nitrite was then determined with solutions of 0.02% *N*-1-naphthylethylenediamine dihydrochloride in 1.5 N HCl and 1% sulfanilic acid in 1.5 N HCl (Smibert and Krieg 1981).

Light and electron microscopy. Light microscopy and electron microscopy were performed as described by Studer *et al.* (1987).

RNA transfer blot analysis. Total soybean root and nodule RNA was isolated as described by Govers *et al.* (1985), denatured in a mixture of dimethyl sulfoxide and glyoxal, and subsequently electrophoresed on 1% agarose gels (Maniatis *et al.* 1982). The RNA was transferred to GeneScreen filters (New England Nuclear, Dreieich, West Germany) and cross-linked to the filters by 1 min of illumination with UV light of 254 nm (Church and Gilbert 1984). Hybridization and washing steps were performed as recommended in the GeneScreen manual. To detect specific mRNAs, the following soybean nodulin cDNA clones served as probes: pGmLb19, a leghemoglobin cDNA clone (Studer *et al.* 1987); pGmENOD2 (Franssen *et al.* 1987); and pGmENOD13 and pGmENOD55 (Gloudemans 1988; B. Scheres, T. Gloudemans, F. Mulckhuyse, H. Bluyssen, A. van Kammen, T. Bisseling, and H. J. Franssen, unpublished).

RESULTS

Phenotype of mutant 3160 on plants. *B. japonicum* mutant 3160 elicited very small, white nodules on soybean (*G. max* 'Williams'), which were unable to fix nitrogen (Fix^-). Only after prolonged growth of the plants (4 wk or longer) did we observe the occasional formation of one larger nodule per plant. The nodules were dispersed over the whole root system, unlike those caused by the *B. japonicum* wild type, which occurred on the main root. The total number of 3160-induced nodules per plant was at least three times higher than that of wild-type-induced nodules. Using light and electron microscopic analysis of 3160-induced nodules (Fig. 1), we could show that the bacteria were able to induce plant cell division, but we

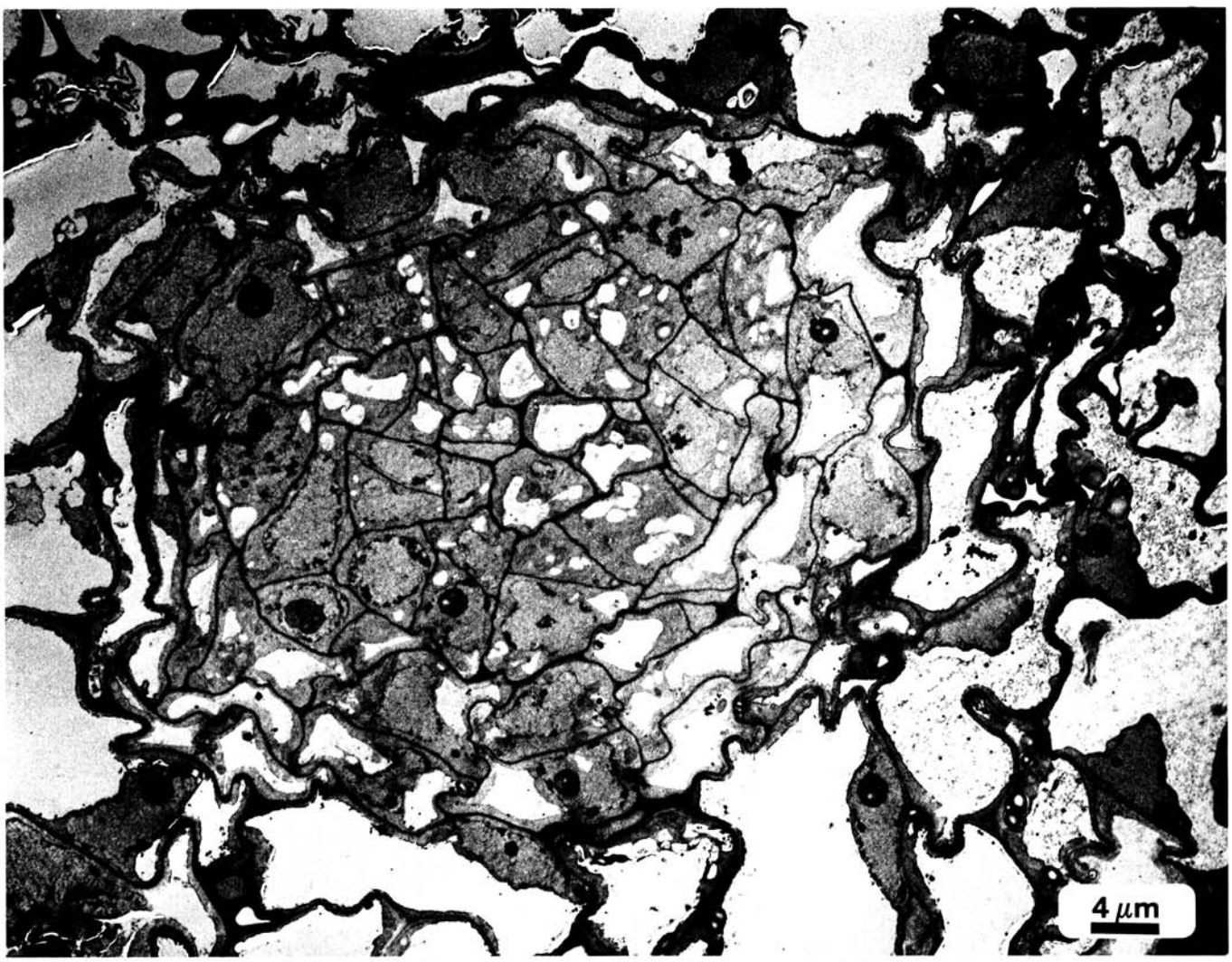

Fig. 1. Electron microscopic examination of a 20-day-old soybean pseudonodule induced by *Bradyrhizobium japonicum* mutant 3160 (×2,500). The sector shows that inner-cortical plant cell division has occurred. At this time after inoculation, nodules infected by the wild type (not shown) are fully occupied by bacteroids.

could not obtain convincing evidence for the presence of infection threads or bacteroids. Also, we could isolate only one to 10 viable bacteria from such nodules (in contrast to the 10^8 bacteria from wild-type-induced nodules), which is consistent with the observed empty-nodule phenotype.

In addition to soybean, nodulation was also tested on three other species of the family Leguminosae known to form a root nodule symbiosis with the *B. japonicum* wild type: *Vigna radiata* (L.) R. Wilczek 'King,' *V. unguiculata* (L.) Walp. 'Red Caloona,' and *Macroptilium atropurpureum* (L.) Urb. 'Siratro.' When inoculated with strain 3160, all three plant species formed tiny, white, Fix^- nodules similar to those on soybean. This shows that the aberrant nodulation ability and nodule morphology induced by strain 3160 are not host-dependent.

Coinoculation experiments. Inoculation of soybean seedlings with two different strains in a 1:1 ratio was performed in order to see whether the defect of mutant 3160 in failing to develop into bacteroids could be overcome by potential helper bacteria as coinoculants. This approach has been applied successfully with Exo^- mutants of *R. meliloti*. The Exo^- mutants alone induced empty nodules on alfalfa, but when alfalfa was coinoculated with Exo^- and Nod^- mutants, normal Fix^+ nodules were formed, from which approximately equal numbers of the Exo^- and Nod^- mutants could be isolated (Müller *et al.* 1988; Klein *et al.* 1988). Thus, the Exo^- mutants "helped" the Nod^- mutants, and the Nod^- the Exo^- mutants, through the complete infection process. Table 2 shows results of coinoculation experiments with *B. japonicum* mutant 3160 plus one of the following strains: a Nod^+Fix^- strain (B3), two Nod^- strains (335 and 168), and the wild type. Although strain 3160 functionally complemented the Nod^- phenotypes of strains 335 and 168, an analysis of the nodule occupancy showed that it failed to develop into bacteroids. This is in striking contrast to the results of coinoculation experiments with the *R. meliloti* Exo^- mutants, and the implications of this finding are discussed further below. Similarly, the two Nod^+ strains (wild type and B3) failed to support nodule invasion by strain 3160 (Table 2).

Early nodulin gene expression. Because mutant 3160 induced empty nodulelike structures on soybean roots, it was of interest to analyze the expression of early nodulin genes in soybean root nodule tissue. Three early nodulin cDNA clones had been isolated from soybean, pGmENOD2, pGmENOD13, and pGmENOD55, all of which encode proline-rich or possibly hydroxyproline-rich proteins, which may be associated with the plant cell wall (Franssen *et al.* 1987; Gloudemans 1988; B. Scheres, T. Gloudemans, F. Mulckhuyse, H. Bluyssen, A. van Kammen, T. Bisseling, and H. J. Franssen, unpublished). The presence of ENOD2 and ENOD13 early nodulins was correlated with nodule morphogenesis, whereas the ENOD55 gene appeared to be fully expressed only after the release of bacteria from the infection thread (Franssen *et al.* 1987; Gloudemans 1988). Figure 2 shows a northern blot analysis of soybean RNA isolated from wild-type- and 3160-induced nodules. RNA from uninfected roots served as a control. Hybridization with a radioactive pGmENOD13 probe detected at least two mRNA species, suggesting that the ENOD13 gene may be part of a small gene family (Gloudemans 1988). In nodules induced by strain 3160 the ENOD2 and ENOD13 mRNA levels were similar to those in wild-type-infected nodules. By contrast, 3160-induced nodules contained only traces of ENOD55 mRNA at day 14 and none at all at day 28 after inoculation. As expected, the late nodulin genes, of which the leghemoglobin genes served as examples (Fig. 2, bottom panel), were not expressed in 3160-induced nodules. In some experiments, minute traces of leghemoglobin mRNA were seen in 28-day-old nodules. We suspect that this originated from one of the few larger nodules that strain 3160 occasionally forms, as described above.

Growth characteristics of strain 3160. In the search for a possible physiologic basis for the observed phenotype of *B. japonicum* mutant 3160 we performed a series of simple growth tests. Compared to its parental strain, 110*rif*15, or to other Tn*5* mutants, strain 3160 was generally characterized by a slower growth rate, both on complex medium (PSY medium) and on minimal media (HM/NH_4^+ medium with either arabinose, mannitol, glucose, fumarate, malate, or succinate as the carbon source, each at 1%, w/v). The most pronounced difference in aerobic growth was observed on HM/arabinose medium when nitrate was the nitrogen source: in liquid culture the wild type grew with a doubling time of 17–18 hr, whereas the doubling time of 3160 was more than 26 hr, and on plates strain 3160 formed only tiny, translucent colonies after prolonged

Table 2. Results of coinoculation of soybean with *Bradyrhizobium japonicum* strains

Strain or strains	Relevant characteristics[a]	Symbiotic phenotype[b]		Number of bacteria isolated from nodules[c]				
		Nod	Fix	Wild type	3160	B3	335	168
Wild type	Rif^r	+	+	10^8				
3160	Rif^r Km^r Bdv^-	+/−	−		5			
B3	Sp^r Km^r *nifB*$^-$	+	−			10^7		
335	Sp^r Km^r *nodA*$^-$	−	−				NA[d]	
168	Sp^r Km^r *nodC*$^-$	−	−					NA
Coinoculation mixture								
3160 + wild type		+	+	10^8	2			
3160 + B3		+	−		1	10^7		
3160 + 335		+	+		1		10^8	
3160 + 168		+	+		10			10^8

[a] Bdv = bacteroid development; Km = kanamycin; Rif = rifampicin; Sp = spectinomycin.
[b] + = Normal nodulation (Nod) or nitrogen fixation (Fix); − = no nodules or no nitrogen fixation; +/− = aberrant nodulation, characteristic of strain 3160.
[c] The bacterial strains were identified by their antibiotic resistance markers.
[d] Not applicable.

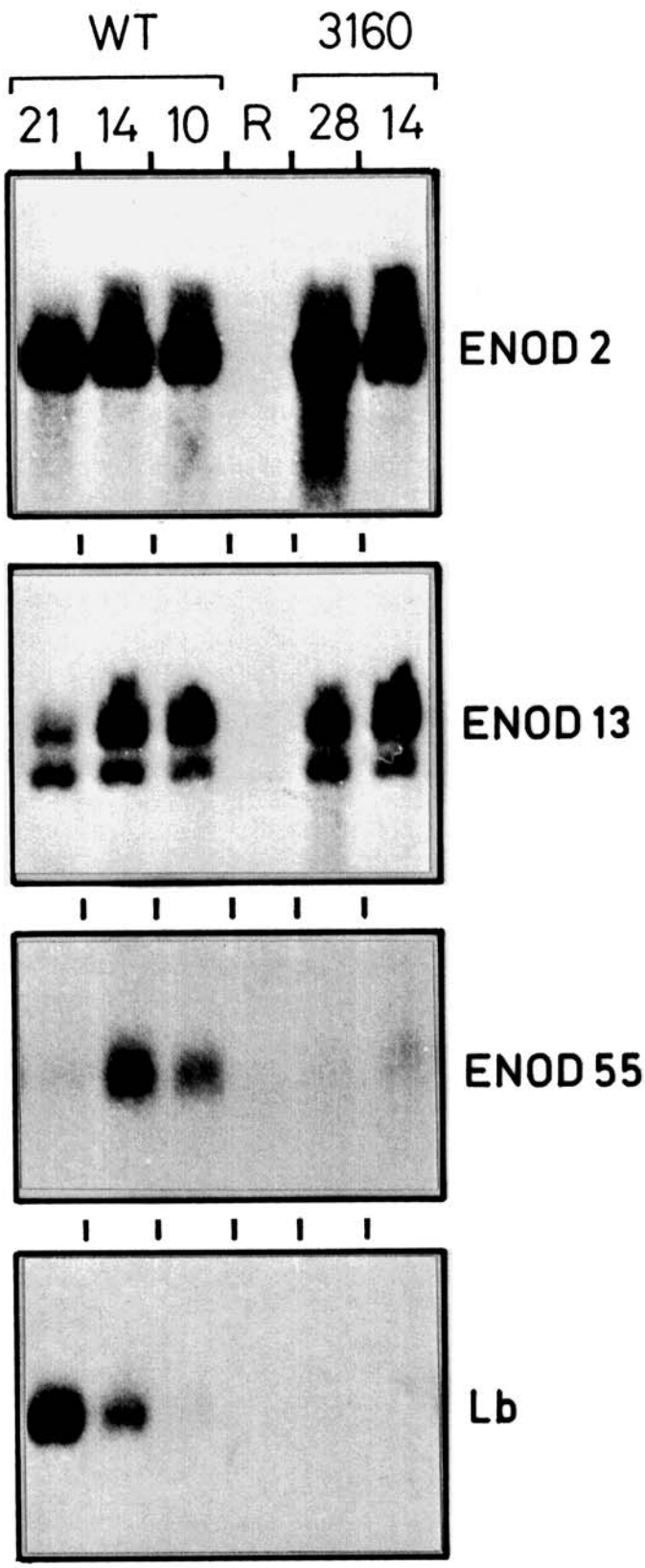

Fig. 2. Northern blot analysis of 15 μg of total RNA isolated from nodules induced by the *Bradyrhizobium japonicum* wild type (WT; left three lanes) and by mutant 3160 (right two lanes). The central lane (R) contains RNA from uninoculated roots. The numbers above the lanes denote the age of the nodules in days after inoculation. Four identical blots were hybridized separately to ^{32}P-labeled soybean cDNA probes specific for ENOD2, ENOD13, ENOD55, and leghemoglobin (Lb) mRNAs.

incubation (more than 2 wk). However, nitrate reductase activity per se was found to be unaffected. Also, strain 3160 was able to grow anaerobically with nitrate as terminal electron acceptor (all data not shown).

The strongly impaired growth of 3160 on minimal HM/arabinose medium plus NO_3^- was overcome completely by the addition of folic acid (10 μg/ml) to the medium. Other vitamins had either no effect (biotin, thiamine, and panthothenic acid) or only an intermediate supplementing ability (riboflavin). This suggested that under these special growth conditions the mutant has a limited, but not absolute, requirement for folic acid. Interestingly, in contrast to the wild type, strain 3160 was found to be resistant to trimethoprim (500 μg/ml) in solid media. Because trimethoprim is an inhibitor of dihydrofolate reductase, the leaky folate auxotrophy of mutant 3160 and the resistance to trimethoprim may have a common basis.

Schwinghamer (1970) described a riboflavin auxotroph of *R. l.* bv. *phaseoli* with a Fix$^-$ phenotype in bean nodules, and he reported that the addition of riboflavin (10 μg/ml) to the plant growth medium helped to overcome this defect. In similar experiments, we added folate, riboflavin, or both, either to the soybean nutrient solution or to the bacterial culture prior to inoculation. However, we observed the same aberrant nodulation behavior of strain 3160 as that observed without vitamin supplements.

Cloning of Tn*5*-containing DNA fragments and of the corresponding wild-type DNA region. It has been shown previously (Regensburger *et al.* 1986) that the genome of mutant 3160 harbors a single Tn*5*-containing *Eco*RI fragment of 7.5 kilobases (kb). This fragment was cloned in pBR329, resulting in plasmid pRJ2300 (Fig. 3). The same fragment was also cloned in pSUP202, giving rise to plasmid pRJ2301 (Table 1). The latter plasmid was used for marker exchange mutagenesis of the *B. japonicum* wild type, which generated a mutant identical in phenotype to the original strain 3160. This proved that the Tn*5* mutation in 3160 is the sole cause of the observed pleiotropic phenotype of this strain.

Plasmid pRJ2300 was used as a probe for colony hybridization of a pLAFR1 cosmid library of *B. japonicum* genomic DNA in *E. coli*. One cosmid, pL44-8C, was thus identified as carrying the corresponding 1.7-kb wild-type *Eco*RI fragment (Fig. 3). Because the 1.7-kb fragment is located at one end of the pL44-8C insert, and no overlapping cosmid was found, we cloned an overlapping Tn*5*-containing *Asp*718 fragment of 19 kb from the 3160 genome into pUC18, resulting in plasmid pRJ2316 (Fig. 3). Appropriate Southern blot hybridizations revealed that the DNA regions cloned in pL44-8C and pRJ2316 are colinear with wild-type *B. japonicum* genomic DNA and that this DNA region does not overlap with any of the *nod*, *nif*, and *fix* regions identified so far in our laboratory (Hennecke *et al.* 1987). Thus, the cloned DNA constitutes a new symbiotically essential gene region from *B. japonicum*.

Approximate location of the symbiotically essential gene region. Two Ω interposon insertions were created: pRJ2325Ω, in a 2.3-kb *Pst*I fragment, and pRJ2330Ω, in a 3.6-kb *Bgl*II fragment obtained from pRJ2316, thereby replacing an internal 0.5-kb *Eco*RI fragment (Fig. 3). These insertions were marker-exchanged into the *B. japonicum* wild type, which generated two additional mutant strains,

2325Ω and Δ2330Ω (Fig. 3). When soybean plants were inoculated with mutant 2325Ω, the mutant had a phenotype indistinguishable from that of mutant 3160. The phenotype of mutant Δ2330Ω, however, was clearly different from that of strain 3160 in that it induced normal nodules in which Fix activity appeared after a long delay: 21 days after inoculation the nodules were still white inside and had only 14% of wild-type Fix activity, whereas after 28 days they were red inside and exhibited 100% of Fix activity. Thus, the mutagenesis data suggest that the insertions in strains 2325Ω and 3160 are located in the same gene or in an operon to which that gene belongs. The deletion in mutant Δ2330Ω possibly lies outside of this putative gene and may affect a second gene.

Interspecies hybridization. When the 1.7-kb wild-type *Eco*RI fragment was used as a hybridization probe, we detected strongly hybridizing bands in genomic DNAs of closely related *Bradyrhizobium* strains (*B. japonicum* 61A76, *Bradyrhizobium* sp. 32H1, and *Bradyrhizobium* sp. from *Parasponia*) and of the nonsymbiotic diazotroph *Rhodopseudomonas palustris* (Molisch) van Niel. Only weak hybridization was found with DNAs of the fast-growing rhizobia *R. meliloti* and *R. l.* bv. *phaseoli* (data not shown).

DISCUSSION

This paper reports on the identification and cloning of a new DNA region from *B. japonicum*, which carries at least one new symbiotically essential gene. This gene is involved in early stages of bacteroid development, because strains with mutations in it (2325Ω and 3160) form bacteroid-free nodulelike structures. The gene may be adjacent to a second bacteroid development gene, because a strain with a deletion in that adjacent region (Δ2330Ω) formed bacteroid-containing Fix^+ nodules only after a delay of about 7 days, compared to the *B. japonicum* wild type.

Mutants similar in nodulation phenotype to strain 3160, for example the Exo^- mutants, have been reported for several other rhizobial species and bradyrhizobial strains (Finan *et al.* 1985; Vandenbosch *et al.* 1985; Stanley *et al.*

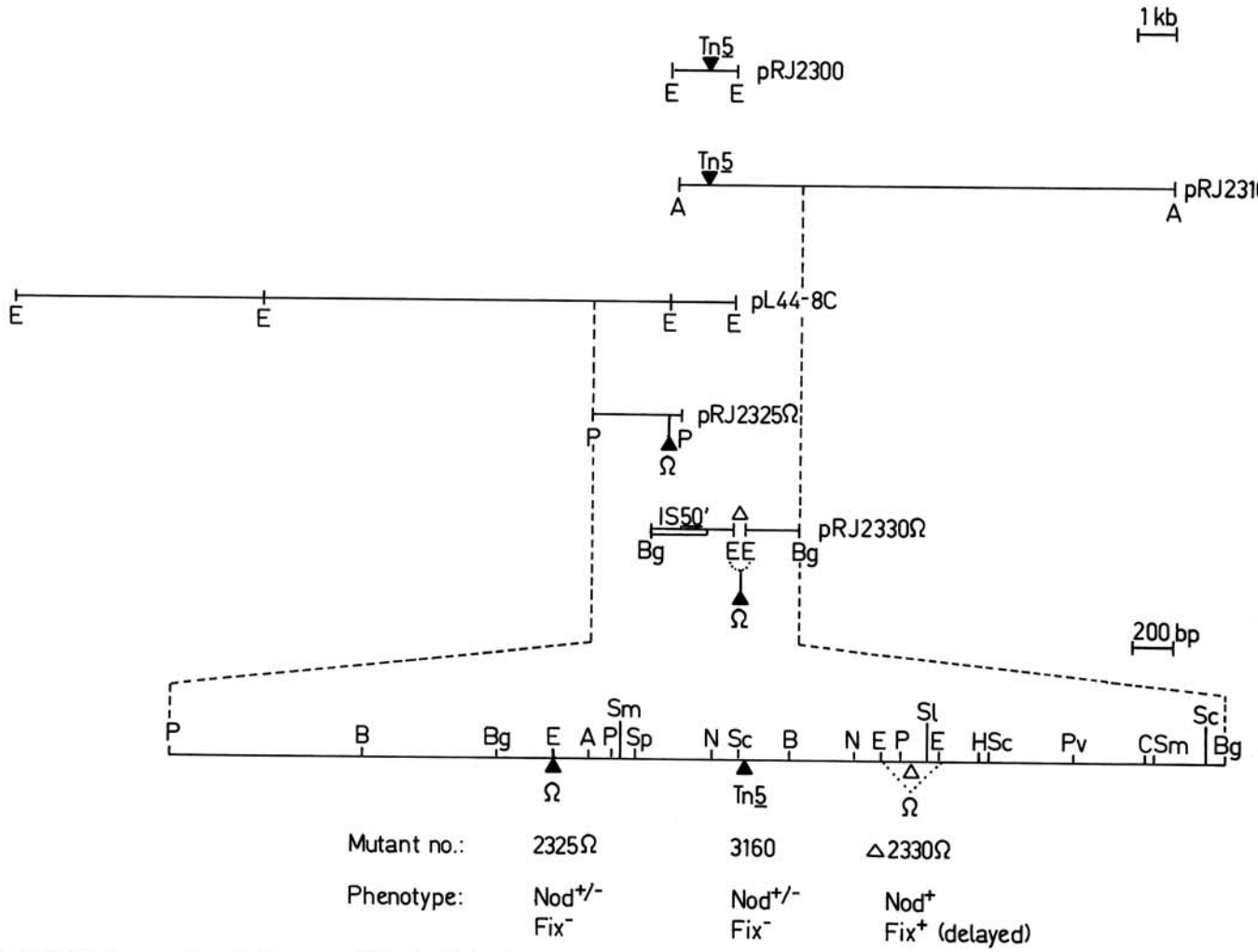

Fig. 3. DNA clones and restriction map of the *Bradyrhizobium japonicum* gene region around the site of the 3160 insertion. Plasmids pRJ2300 and pRJ2316 are Tn*5*-containing subclones isolated from mutant 3160. Cosmid pL44-8C is from a wild-type gene bank. Plasmids pRJ2325Ω and pRJ2330Ω contain omega cassette insertions and were used for marker exchange. A detailed restriction map of a 6.5-kb genomic region is given below the clones and shows the sites of the three mutations (triangles) and the phenotypes of the corresponding mutants ($Nod^{+/-}$ stands for the characteristic empty-nodule phenotype). Restriction sites are abbreviated as follows: A, *Asp*718; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; and Sp, *Sph*I.

1986; Dylan *et al.* 1986; Morrison and Verma 1987; Leigh *et al.* 1987; Müller *et al.* 1988; Putnoky *et al.* 1988). In this state of knowledge of our own as well as all the other mutants described, however, it is impossible to determine whether or not analogous genes are affected in the different systems. Nucleotide sequence information is available for only two genes involved early in bacteroid development. One is the *ndvA* gene of *R. meliloti*, a homologue of the *chvA* gene of *Agrobacterium tumefaciens* (Smith and Townsend) Conn, which has been implicated in the export of β-(1→2)-glucan from the cells (Stanfield *et al.* 1988). The other sequenced gene is an unidentified reading frame (URF) of 249 codons from *B. japonicum* strain 61A76 (Morrison and Verma 1987) in which a Tn*5* insertion renders the mutant unable to undergo endocytosis (release from infection thread). We have established the nucleotide sequence of a 262-bp *Sac*I-*Bam*HI fragment spanning the site of the Tn*5* insertion in mutant 3160 and clearly found no homology of DNA and deduced protein sequences (in all six frames) to *ndvA* or URF (M. Göttfert and S. Rossbach, unpublished data).

Our search for a possible biochemical basis of the mutant phenotype has not yielded conclusive results. Perhaps the most interesting aspect is the finding that strain 3160 exhibits a leaky folate auxotrophy when grown on minimal medium with nitrate as the sole nitrogen source. However, it is not known why this partial auxotrophy occurs only under this special growth condition. Rhizobial vitamin, purine/pyrimidine, and amino acid auxotrophs possessing a nodulation-defective phenotype have been described previously (Schwinghamer 1970; Schwinghamer 1977; Sadowsky *et al.* 1986; Bassam *et al.* 1987; Noel *et al.* 1988; Kim *et al.* 1988). In many, but not all, of these cases the addition of the required metabolite in the plant inoculation test led to a restoration of nodulation. Similar experiments to restore the nodulation capability of mutant 3160 by supplementing folate have been unsuccessful. Thus, the question of whether or not folate starvation is the explanation for the failure of mutant 3160 to proliferate in symbiotic association with soybean or other compatible host plants remains unanswered.

Several studies have reported that late nodulins are not expressed in nodules lacking infection threads and intracellular bacteria in soybean (Morrison and Verma 1987) and in alfalfa (Dunn *et al.* 1988; Putnoky *et al.* 1988). What is new and different with mutant 3160 is the finding that it uncouples the expression of the early nodulin genes ENOD2 and ENOD13 from that of another early nodulin gene, ENOD55, that is mRNA of the first two nodulin genes is detectable in the pseudonodules induced by 3160, whereas ENOD55 mRNA synthesis is strongly reduced. Only very soon after inoculation a trace amount of ENOD55 mRNA is visible, which may have been synthesized in response to just a few bacteria entering the root hair cells. This assumption is consistent with our finding that we could isolate only one to 10 bacteria from a 3160-induced nodulelike structure. In conclusion, the data show that the degree of ENOD55 expression may be correlated strictly with the number of invading microsymbionts present after infection.

Mutant 3160 is unable to invade the nodule cells even together with other *B. japonicum* as coinoculants. The fact that 3160 itself restores soybean nodulation by *nodA* and *nodC* mutants shows that the signal production conferred by the common *nod* genes (Schmidt *et al.* 1988) is intact in strain 3160. In contrast to results of mixed-inoculation experiments involving *R. meliloti* Exo$^-$ mutants (Müller *et al.* 1988; Klein *et al.* 1988), neither the *B. japonicum* wild type nor *nif* and *nod* mutants had the ability to restore nodule invasion by strain 3160. This makes it unlikely that the gene affected in 3160 is involved in the synthesis of a diffusible signal molecule, because such a putative signal should have been produced by the coinoculant bacteria having no lesion in the DNA region around the site of the 3160 mutation. It seems more likely that strain 3160 suffers from a severe growth defect, in particular after having entered the root hair cells, and, therefore, cannot compete with the coinoculant bacteria.

The capability of strain 3160 to restore the nodulation of soybean by *nodA* and *nodC* mutants bears another interesting implication. This result shows that the physical presence of *nodABC*$^+$ bacteria throughout the complete nodulation process is not an essential prerequisite for helping the Nod$^-$ coinoculant succeed in generating an effective symbiosis. This can be interpreted to mean either that the common *nod*-dependent signal generation (Schmidt *et al.* 1988) is required only in a very early stage of infection or that the amount of signal produced by the few 3160 cells associated with a pseudonodule suffices to phenotypically cross-complement the *nodA* and *nodC* lesions through the entire infection cycle.

In conclusion, the phenotypic and genetic characterization of mutant 3160 has led to the identification of a new *B. japonicum* gene, which plays an important role in one of the earliest possible steps in bacteroid and root nodule development. The function of the newly detected gene may not necessarily be an exclusive one in legume nodulation, because a homologous DNA region appears to exist in *Rhodopseudomonas palustris*, which is a close relative of *B. japonicum* (Hennecke *et al.* 1985) but not a root nodule symbiont.

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