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Identification of a Locus Upstream from the Hydrogenase Structural Genes That Is Involved in Hydrogenase Expression in *Bradyrhizobium japonicum*†

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A locus involved in the expression of the uptake hydrogenase system of *Bradyrhizobium japonicum* was identified adjacent to genes encoding the hydrogenase subunits. A cloned fragment of DNA was used to complement to autotrophy a Hup$^-$ putative regulatory mutant of *B. japonicum*. The mutant strain lacked hydrogenase activity and synthesized low levels of the large subunit of hydrogenase as determined by Western gels. Tn*5*-induced mutagenesis located the region within the fragment which was necessary for complementation of the mutant phenotype. The locus identified is adjacent to that encoding the small subunit of hydrogenase; its right border is <0.5 kilobase upstream from the hydrogenase transcriptional start site, and its left border is between 1 and 2.5 kilobases from that start site. However, the locus is outside the region previously shown to contain *hup*-related genes of *B. japonicum*. Thus, the localization of this gene describes a previously unidentified *hup*-related gene on a region of DNA not previously shown to contain *hup*-specific DNA.

Strains of *Bradyrhizobium japonicum* which contain an uptake hydrogenase (Hup$^+$) can grow chemoautotrophically with H_2 and CO_2 as the sole energy and carbon sources, respectively (13, 22). The ability to grow chemoautotrophically is a means of assaying the Hup phenotype of *B. japonicum* (13, 22, 27) and has been used to isolate mutants lacking such an ability to oxidize hydrogen (Hup$^-$) (16, 23, 27, 28).

The Hup$^-$ mutant SR139 was isolated as a mutant unable to grow chemoautotrophically (Aut$^-$) and was subsequently shown to lack hydrogen uptake activity either in free-living culture or as bacteroids, with either oxygen or methylene blue provided as electron acceptor (34). Symbiotically, SR139 has been shown to be both Hup$^-$ and Nif$^-$ (27). Extracts from strain SR139 appear to lack both subunits of nitrogenase, as well as both nitrogenase and hydrogenase activities (34). The high reversion rates and the ubiquity of reversion simultaneously to a Hup$^+$ and Nif$^+$ phenotype indicate that the mutation is the result of a single lesion. Similar Hup$^-$ Nif$^-$ mutants of *B. japonicum* with similar reversion characteristics have been described recently (26). Based on the pleiotropic effects caused by what appears to be a single lesion, strain SR139 is probably a regulatory mutant.

The cosmid pSH22, containing 23.2 kilobases (kb) of *B. japonicum* DNA, can complement SR139 to an Aut$^+$ phenotype at a frequency of 1.0 per cosmid transfer (15). The cosmid-containing transconjugants can be derepressed for hydrogenase activity in free-living culture, and nodules from plants inoculated with the transconjugants have had detectable levels of nitrogenase activity (15). Some nitrogenase activity has also been detected ex planta in the transconjugants (15), although derivatives of strain SR are not easily inducible for nitrogenase activity ex planta (1). Thus, the single cosmid pSH22 has been shown to complement the defects in both nitrogenase and hydrogenase activities of SR139.

In the course of the experiments, cosmid pSH22 was successively subcloned to localize the region involved in the defect of strain SR139. Tn*5*-induced mutagenesis, mapping, and complementation analysis were used to pinpoint the locus involved at least in the Hup$^-$ phenotype of SR139. The locus was mapped in relation to the previously identified cluster of *hup*-related genes of *B. japonicum* (14, 15), including the hydrogenase structural genes (38, 43, 44).

MATERIALS AND METHODS

Chemicals. Antibiotics and restriction enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim Biochemicals, Indianapolis, Ind. Gases were obtained from Arundel Sales and Service, Baltimore, Md. Nickel chloride was Puratronic grade, from Johnson Mathey Chemists Limited, Hertfordshire, England. All other chemicals were reagent grade or purer.

Bacterial strains and plasmids. *B. japonicum* SR is an Sm^r derivative of USDA 122 (30). Strain SR139 is a Nif$^-$ Hup$^-$ mutant of strain SR (27). The *Escherichia coli* host used for genetic manipulations was strain HB101 (3). Cosmid pSH22 contains 23.2 kb of *B. japonicum* DNA in the broad-host-range vector pLAFR1 (10) and has been described previously (15). The vectors used for subcloning were the broad-host-range vector pRK290 (7) and its derivative, pRK404 (6).

Media and cell growth. *B. japonicum* was cultured on modified Bergersens (BM) agar plates (2) supplemented with the following antibiotics when necessary: kanamycin (Km) (100 μg/ml), streptomycin (Sm) (100 μg/ml), and tetracycline (Tc) (80 μg/ml). In liquid cultures of *B. japonicum*, BM (2) contained 5 μM $NiCl_2$ and antibiotics when necessary. When used, the antibiotics were supplied at the following concentrations: 80, 50, and 50 μg of tetracycline, kanamycin, and streptomycin per ml, respectively.

E. coli cultures which contained cloned plasmids were grown in LB, as described before (31), supplemented with selective antibiotics. The medium was supplemented with tetracycline at 20 μg/ml for growth of transconjugant cul-

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tures containing subclones in vector pLAFR1 (10), pRK290 (7), or pRK404 (6) or with 20 μg of kanamycin per ml for HB101 containing plasmid pRK2013 (9). For transconjugants containing Tn*5*-mutated plasmids, both tetracycline (20 μg/ml) and kanamycin (50 μg/ml) were used. For most experiments, *B. japonicum* and *E. coli* were grown to mid-log phase (optical density of 0.5 at 540 and 550 nm, respectively). Cell numbers were routinely estimated by measurements of optical density. A_{540} was proportional to cell number over most of the range of log-phase growth for *B. japonicum*.

Derepression of hydrogenase. After being grown to a cell density of 4×10^8 to 6×10^8 cells per ml, bacteria were harvested by centrifugation, washed, and suspended in No-carbon medium (27) containing 5 μM $NiCl_2$. When transconjugants were derepressed, selective antibiotics were present during growth, but were absent from the derepression medium. The cell density at the start of derepression was approximately 4×10^8 cells per ml. Cells were derepressed under 1.0% oxygen, as described previously (29, 40), for 24 to 40 h. Hydrogenase activity was assayed amperometrically as reported earlier (12, 41), with O_2 (saturating levels) as the electron acceptor. H_2 uptake values are the average of at least two independent determinations.

Preparation of membranes for Western gel analysis. Membranes were prepared from derepressed cells by lysis through a French pressure cell as described previously (35). Hydrogenase activity of wild-type whole cells was 180 nmol/h per 10^8 cells but was not detectable in strain SR139. The membranes were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and immunostained for hydrogenase subunits as described before (33, 39). Each lane contained 3 μg of protein, and duplicate gels were stained with silver (32) to confirm that the lanes for both strains were loaded with equal amounts of protein.

Subcloning pSH22. pSH22 (15) DNA was digested with *Eco*RI. The total digestion mixture was treated with phosphatase (31), with proteinase K included in the stop buffer. The phosphatase-treated DNA was ligated to *Eco*RI-digested pRK290 (7). Ligations were performed as described before (31). The ligated DNA was used to transform *E. coli* HB101, and Tc^r colonies were picked. Clones containing inserts were identified by agarose gel electrophoresis of restriction enzyme-digested DNA. Four of the five *Eco*RI fragments of pSH22 were cloned in this manner. The fifth was cloned after isolation of the *Eco*RI fragment from a low-melting-temperature agarose gel (31).

Subcloning pDN11. pDN11 DNA was partially digested with *Bgl*II, and the fragments were ligated as a mixture to *Bgl*II-cleaved, phosphatase-treated pRK290 DNA. Transformed HB101 cells were selected and analyzed as described above. Because a partial digest of pDN11 was used, each single *Bgl*II fragment plus larger fragments containing internal *Bgl*II sites were cloned (see Fig. 3). Because pDN11 was an *Eco*RI insert in the *Eco*RI site of pRK290, the rightmost 1.6 kb of *B. japonicum* DNA on pDN11 was not cloned. In addition, the leftmost 2.3-kb fragment cloned consisted of pDN11 insert DNA (1.2 kb) plus DNA from vector pRK290 (1.1 kb).

Subcloning pRY12. Plasmid DNA was digested with *Bam*HI, and fragments were separated by agarose gel electrophoresis. DEAE-nitrocellulose membrane strips (NA 45; Schleicher & Schuell, Keene, N.H.) were used in the band interception technique (8, 42) to capture the 3.1- and 3.2-kb *Bam*HI fragments of pRY12. The bands were isolated as a mixture of the two sizes, mixed with *Bam*HI-digested, phosphatase-treated pRK404 (6), and cloned and selected as described above.

Plasmid DNA isolation. Plasmid DNA was routinely isolated from minilysates of cells (17), and the suspended DNA was further purified by precipitation with ammonium acetate and ethanol (31). When large volumes of plasmid DNA were required, plasmid isolations were performed by cesium chloride gradient centrifugation (31).

Filter matings. Subcloned plasmids were conjugated from *E. coli* to *B. japonicum*, using the triparental filter-mating technique (5) with the modifications described by Hom et al. (16). Transconjugants were selected on BM (2) agar containing 5 μM $NiCl_2$ and 80 μg of tetracycline per ml. For selection of Tn*5*-mutated transconjugants, 50 μg of kanamycin per ml was also present. Recipient *B. japonicum* cells, which were Tc^r (and Km^r when applicable), were streaked on No-carbon agar medium (27) and incubated in a microaerophilic environment as described previously (29) to test for complementation to autotrophy. Some 500 colonies from each mating were streaked. Alternatively, individual transconjugants were picked, grown in culture, and derepressed for hydrogenase activity.

Tn*5* mutagenesis. Plasmid pRY12 was mutagenized by Tn*5* insertion, using a λNK467::Tn*5* vector, essentially as described by Ruvkun and Ausubel (37). The λNK467::Tn*5* was kindly provided by Frederick Ausubel. The multiplicity of infection was 1.0. Samples of the infected cells were plated onto LB containing tetracycline and kanamycin and incubated at 30°C. Plasmid DNA isolated from the antibiotic-resistant recipients was used to retransform HB101, and again tetracyline- and kanamycin-resistant colonies were selected. Plasmid DNA from these colonies was isolated and analyzed by restriction enzyme digestions and gel electrophoresis, using the restriction map of pRY12 generated during the study as well as the published restriction map of Tn*5* (18).

RESULTS

Characterization of SR139. Previous results had shown that strain SR139 lacked hydrogenase and nitrogenase activities, but the mutant could be complemented to Hup^+ Nif^+ by cosmid pSH22, which contains genes involved in H_2-oxidizing activity. To analyze this mutant further, SR139 extracts were assayed for individual hydrogenase subunits as detected by subunit-specific antiserum. The results (Fig. 1) showed that the mutant synthesized less 65-kilodalton hydrogenase subunit than did the wild type, but it did synthesize a detectable level. This result was observed in three separate experiments, and control silver staining of duplicate gels confirmed that approximately equal amounts of protein were loaded for wild type and SR139.

The results with the small-subunit-specific antiserum were not conclusive; no reaction was detected with the mutant extracts, but wild-type extracts also reacted weakly. Thus, strain SR139 appeared either to lack the small subunit or at least to contain less than did the wild-type strain. The small-subunit antiserum has been noted previously to be a less efficient probe for hydrogenase than the large-subunit antiserum (33, 39).

Subcloning pSH22. Cosmid pSH22 contained a large fragment of *B. japonicum* DNA. To localize the region involved in the defect, the clone was restriction mapped, using electrophoretic analysis of partial and complete digestions with *Eco*RI. The five *Eco*RI fragments of pSH22 were subcloned into the broad-host-range vector pRK290 (7) and

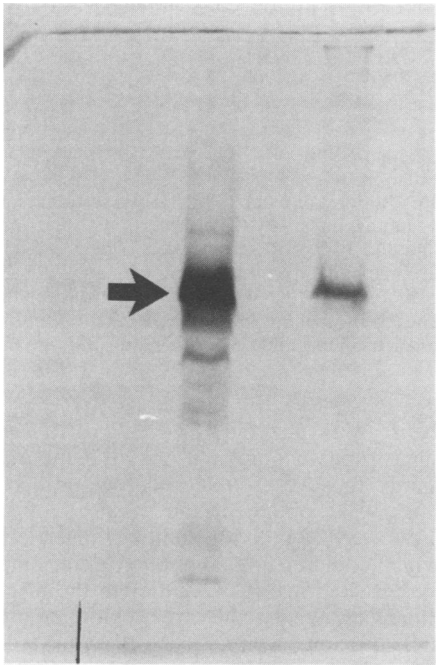

FIG. 1. Western gel analysis of the large subunit of hydrogenase from membranes obtained from wild-type (left) and SR139 (right) lanes. Arrow shows the location of the large subunit of hydrogenase.

used to transform *E. coli* HB101. Figure 2 shows the map of pSH22 as well as the resulting *Eco*RI subclones. Each of the subclones was individually mated from *E. coli* into *B. japonicum* SR139 by using the triparental filter-mating technique. Tetracycline-resistant *B. japonicum* recipients were selected and screened for their ability to grow autotrophically by streaking them on carbon-free plates and incubating them microaerobically (1.0% O_2) (24, 27). Table 1 details the efficiency of each mating (Tc^r per recipient) and the frequency of complementation to autotrophy of SR139 by each of the subclones.

pDN11 complemented SR139 to autotrophy at a frequency of 1.0 Aut^+ per plasmid recipient when 500 colonies of each subclone were tested. No other subclone complemented to autotrophy even 1 of the 500 transconjugants tested. Thus,

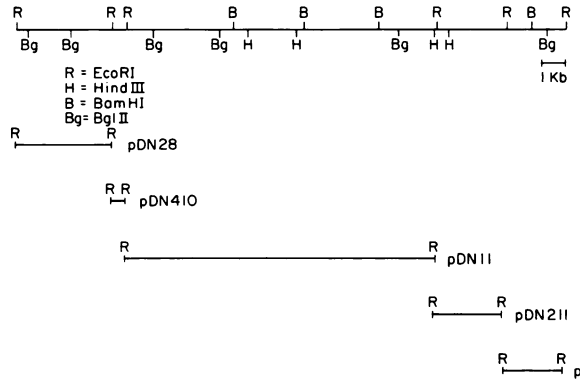

FIG. 2. Restriction map and *Eco*RI subclones of cosmid pSH22 (15). The 13.2-kb *Eco*RI fragment, designated pDN11, was used for further subcloning in these experiments. Procedures for mapping and subcloning are described in the text.

TABLE 1. Complementation of SR139 by subclones of pSH22

Subclone	Insert size (kb)	Tc^r/recipient[a]	Aut^+/Tc^r[b]
pDN11	13.2	3.5×10^{-4}	1.0
pDN28	4.0	2.0×10^{-5}	$<2 \times 10^{-3}$
pDN38	2.5	3.9×10^{-5}	$<2 \times 10^{-3}$
pDN410	0.6	1.5×10^{-5}	$<2 \times 10^{-3}$
pDN211	3.0	4.8×10^{-3}	$<2 \times 10^{-3}$
pSH22	23.3	4.5×10^{-3}	1.0
pRK2013[c]		4.1×10^{-8}	ND
SR139		2.5×10^{-8}	$<2 \times 10^{-3}$

[a] The efficiency of mating, the number of transconjugants relative to the total number of recipient cells, measured by cell growth on selective plates.
[b] The frequency of complementation to autotrophy, measured by the ability of the cells to grow chemoautotrophically. ND, Not determined.
[c] Control. Helper plasmid (pRK2013) alone mated into SR139.

the gene on pSH22 responsible for complementing SR139 to autotrophy resided within the 13.2-kb *Eco*RI fragment of DNA on subclone pDN11.

Subcloning pDN11. Because pDN11 was large enough to encode numerous genes, it too was subcloned. A more complete restriction map was generated by using combinations of the enzymes *Bam*HI, *Hin*dIII, and *Bgl*II (Fig. 2), and mapped locations on pSH22 were confirmed by independent restriction mapping of each of the *Eco*RI subclones of pSH22. Fragments generated by a partial *Bgl*II digestion of pDN11 were ligated en masse to pRK290. The *Bgl*II fragments of pDN11 DNA were 2.8, 7.6, and 2.3 kb (Fig. 3). The first two were wholly derived from pDN11 insert DNA; the 2.3-kb fragment contained 1.2 kb of pDN11 insert and 1.1 kb of pRK290. The rightmost 1.6-kb *Bgl*II-*Hin*dIII fragment of pDN11 was not subcloned.

Subclones were obtained that contained each individual insert as well as larger fragments derived from the partial digestion. The subclones, designated the pRY series, were individually mated into SR139, and again tetracycline-resistant recipients were screened for ability to grow autotrophically (Table 2). All subclones which contained the 7.6-kb

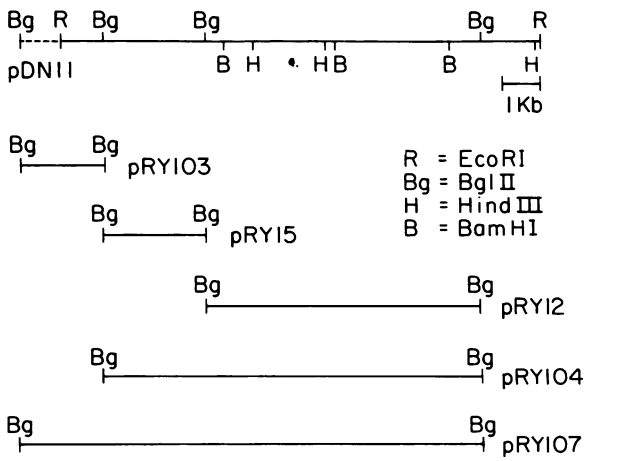

FIG. 3. Restriction map and *Bgl*II subclones of pDN11. The fragment used for subcloning was the 13.2-kb *Eco*RI fragment designated pDN11 in Fig. 2. The dashed line represents the portion of the vector (pLAFR1 [10]) cloned along with the adjacent portion of pDN11 to create pRY103. Procedures for restriction mapping and subcloning are given in the text.

TABLE 2. Complementation of SR139 by subclones of pDN11

Subclone	Insert size (kb)	Tc^r/recipient[a]	Aut$^+$/Tc^r[b]
pRY12	7.6	9.3×10^{-3}	1.0
pRY15	2.8	3.2×10^{-3}	$<5 \times 10^{-3}$
pRY103	2.2	7.6×10^{-3}	$<5 \times 10^{-3}$
pRY104	7.6 + 2.8	8.6×10^{-3}	1.0
pRY107	7.6 + 2.8 + 2.2	1.5×10^{-2}	1.0
pDN11	13.2	6.6×10^{-3}	1.0
pRK2013[c]		$<8 \times 10^{-8}$	ND
SR139		1.7×10^{-8}	$<5 \times 10^{-3}$

[a] The efficiency of mating, the number of transconjugants relative to the total number of recipient cells, measured by cell growth on selective plates.
[b] The frequency of complementation to autotrophy, measured by the ability of the cells to grow chemoautotrophically. ND, Not determined.
[c] Control. Helper plasmid (pRK2013) alone mated into SR139.

*Bgl*II fragment complemented SR139 at a frequency of 1.0 Aut$^+$ per plasmid recipient, while no subclone lacking that fragment complemented SR139 to autotrophy (Aut$^+$/Tc^r, $<2 \times 10^{-3}$). In the SR139(pRY12) transconjugants, substantial hydrogenase activity, comparable to wild-type levels, could be derepressed. The fragment could thus complement the Hup$^-$ phenotype of SR139 to Hup$^+$. None of the Aut$^-$ transconjugants could be derepressed for hydrogenase activity. The 7.6-kb piece alone was able to complement SR139; therefore, the gene of interest resided on that fragment.

Subcloning pRY12. The 7.6-kb fragment was also large enough to encode multiple genes. Therefore, to pinpoint the location of the gene, *Bam*HI fragments encompassing that region were used for further subcloning into vector pRK404 (6). The *Bam*HI fragments of pRY12 were 3.1 and 3.2 kb (Fig. 3). By using the band interception technique, the two bands were isolated together and ligated to pRK404 as a mixture. Both individual inserts were represented among the subclones obtained, and one subclone was also obtained which contained both the 3.2- and 3.1-kb fragments. Subsequent restriction mapping determined that the fragments were not in their usual orientation relative to each other, although their exact orientation could not be determined from the restriction map or from digestion with several additional restriction enzymes (*Pvu*I and *Xho*I). Each subclone was mated individually into SR139 (not shown), and the transconjugants were tested for complementation to autotrophy. Neither of the *Bam*HI fragments nor the combination of the two, out of order, complemented SR139 to autotrophy. The results obtained with the *Bam*HI fragment subclones indicated that one of the three *Bam*HI sites was situated within the locus determining the SR139 mutant phenotype.

Tn*5* mutagenesis. Tn*5* insertions into pRY12 were used to define the region necessary for the complementation of SR139. HB101 containing plasmid pRY12 was mutagenized with Tn*5*, using a λNK467::Tn*5* vector (37). DNA was collected and pooled from all plaques which resulted from the mutagenesis and was used to retransform HB101, with the Tc^r Km^r colonies which appeared representing those cells containing a Tn*5*-mutated pRY12 plasmid. Approximately 700 such Tn*5* mutants were isolated. Some 94 randomly chosen transposon mutants were picked and analyzed further with restriction enzymes. Of the 94, 36 had Tn*5* insertions in the pRK290 vector, 7 appeared to have multiple or complex insertions, and 8 could not be identified; these were not studied further.

The 43 remaining Tn*5* mutants, which contained Tn*5* in the pRY12 insert DNA, were mapped by using restriction enzyme analysis. With enzymes *Bgl*II, *Eco*RI, *Bam*HI, and *Hin*dIII, singly and in combinations, the mutations were mapped mainly to several clustered regions of the DNA. Some siblings were obtained; the majority of sites were distinct. There were 23 individual sites of insertion spanning the DNA which could be unambiguously determined, and 20 of those are depicted in Fig. 4. Although the insertions did not cover the DNA evenly, the three regions of interest, the three *Bam*HI junctions, were each well saturated by insertions. In addition, the gaps left unmutagenized were <1.5 kb in size. The Tn*5*-mutated plasmids were referred to as the pSB series, with the clone number the same as the mapped insertion number.

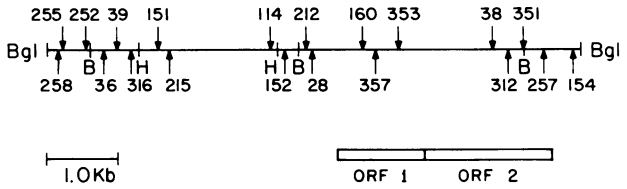

FIG. 4. Restriction map sites of Tn*5* insertions into pRY12. pRY12 was mutagenized by Tn*5* insertions, and mutagenized clones, designated the pSB series, were obtained. The sites of insertion were mapped by restriction enzyme analysis. Each site of insertion is labeled with the number of the clone containing that insertion. The open reading frames (ORF) for the hydrogenase structural genes, as determined by sequencing (38), are indicated for comparison.

Representative clones of 20 of the 23 sites of insertion were picked, and plasmids were mated into SR139 in the usual number. The Tc^r Km^r *B. japonicum* colonies were picked, grown in individual cultures, and derepressed for hydrogenase activity. The efficiencies of mating were within the normal range (not shown), and the standard controls, plating plasmid-free recipients or recipients containing only the helper plasmid, were performed. Duplicates of each sample, at least, were assayed amperometrically for hydrogenase activity (Table 3). The absolute activities are given, as well as their percentages of the control level of activity (SR139 containing pYR12). The level of hydrogenase activity obtained in those control cells was not necessarily representative of the level of activity which would have been obtained in wild-type cells (although the level of hydrogenase activity observed, 154 nmol/h per 10^8 cells, was on the high end of the normal range for strain SR). Thus, comparisons of the abilities of different Tn*5*-mutated plasmids to complement SR139 were possible, but direct comparisons should not be made to wild-type Hup$^+$ cells.

Tn*5* insertions usually had a slightly deleterious effect on derepression of hydrogenase. This effect was seen even in the cells containing Tn*5* inserted far from the hydrogenase genes (data not shown), as well as in cells which did not contain Tn*5* insertions but which were under double antibiotic selection. There was some variability in the levels of hydrogenase activity obtained in the different Tn*5*-containing transconjugants, but the large number of closely clustered insertions distinguished such variability from real differences in hydrogenase activity. Insertion in only one area consistently resulted in dramatic loss of hydrogenase activity. When Tn*5* was inserted in that area, no more than 8% of control levels of hydrogenase activity were observed.

The site was in fact one of the three *Bam*HI junctions predicted by complementation assays to lie within the region of interest. Tn*5* insertions at or around the central *Bam*HI site of pRY12 eliminated the ability of that clone to comple-

TABLE 3. Hydrogen uptake activities of SR139 transconjugants containing Tn5-mutated plasmid derivatives of pRY12

Transconjugant	Hydrogenase activity (nmol/h per 10^8 cells)[a]	% of control[b]
SR139(pSB258)	170	110
SR139(pSB255)	129	84
SR139(pSB252)	165	107
SR139(pSB36)	181	118
SR139(pSB39)	180	118
SR139(pSB316)	44	29
SR139(pSB151)	99	64
SR139(pSB215)	131	85
SR139(pSB114)	7	5
SR139(pSB152)	8	5
SR139(pSB212)	4	3
SR139(pSB28)	12	8
SR139(pSB160)	102	66
SR139(pSB357)	86	56
SR139(pSB353)	104	68
SR139(pSB38)	152	99
SR139(pSB312)	119	77
SR139(pSB351)	104	68
SR139(pSB257)	126	82
SR139(pSB154)	94	61
SR139(pRY12)	154	100

[a] Hydrogenase activity was measured amperometrically. Values represent the average of at least two independent determinations, each from two independent derepressions of hydrogenase activity.

[b] Hydrogenase activity relative to that of SR139 containing pRY12.

ment SR139 to a Hup$^+$ phenotype. In addition, the site included sufficient DNA to encompass a moderately sized gene. Thus, some portion of the gene responsible at least for the Hup$^-$ phenotype of SR139 spanned that central *Bam*HI junction. The possible outer limits of the gene were defined by the Tn5 insertions 160 and 215, since SR139 transconjugants containing those insertions (pSB160 and pSB215) could be derepressed for hydrogenase activity.

Assays of nitrogenase activity. Numerous attempts were made to ascertain whether the same locus was involved in the Nif$^-$ phenotype of SR139. Plasmid-containing transconjugants were used to inoculate soybeans, but in the absence of antibiotic selection the plasmid was not retained through the period of nodulation. Nodule isolates were no longer Tc^r. When antibiotic selection was used (added to the plant nutrient solution), nodules were not formed. Ex planta assays were also attempted. However, the conditions for induction of nitrogenase in free-living culture (11) are not optimal for induction of the enzyme in derivatives of strain SR, and therefore consistent and reliable results could not be obtained with strain SR139. Nor were attempts to cross the Tn5-mutated region into the chromosome successful. Therefore, whether the locus was also involved in the determination of the Nif phenotype could not be assessed.

DISCUSSION

The apparently single lesion of mutant SR139 had pleiotropic effects. Studies of free-living cells and bacteroids have indicated that SR139 lacks activity for both nitrogenase subunits and the hydrogenase enzyme. Thus, SR139 has been defined as a putative regulatory mutant. Additional characterization of the mutant in this study showed that SR139 synthesizes some hydrogenase enzyme (large subunit) of the correct molecular size but greatly reduced in amount compared with the wild type.

Previous studies had indicated that the gene or genes responsible for complementing the Hup$^-$ portion of the phenotype resided on a 23.2-kb stretch of *B. japonicum* DNA (15). The present study pinpointed the central *Bam*HI site of clone pRY12 as the locus involved in the Hup$^-$ defect of SR139. While pRY12 could complement SR139 to a Hup$^+$ phenotype, pRY12, which contained Tn5 inserted at or between sites 114 and 28 (Fig. 4), could not do so. Because the Tn5-mutated plasmids pSB215 and pSB160 could still complement SR139 to a Hup$^+$ phenotype, they determined the absolute outer limits of the putative regulatory locus.

The stretch of DNA which includes cosmid pSH22 has been shown to encode multiple *hup*-related transcription units (14, 15). The genes encoding the two subunits of hydrogenase have been located on that region of DNA (38, 43, 44), as have one or more additional genes (15, 16, 20, 21, 43). The transcriptional start site and open reading frames of the hydrogenase structural genes have been determined by sequencing (38) and are indicated for comparison on Fig. 4. The complementation data obtained by Tn5 mutagenesis in this study indicated that the defect of SR139 is not located in the region encoding the structural genes of hydrogenase. Tn5 inserted into the region of pRY12 which encodes the structural genes of hydrogenase (mutations 160 to 257, inclusive) did not eliminate the ability of the plasmid to complement SR139. The locus defective in SR139 is adjacent to that encoding the small subunit of hydrogenase; its right border is <0.5 kb upstream from the hydrogenase transcriptional start site, and its left border is between 1 and 2.5 kb from that start site. It is of interest to note that a gene adjacent to the hydrogenase structural genes of *Methanobacterium thermoautotrophicum* probably encodes a polyferredoxin, a peptide containing multiple, tandemly repeated, bacterial, ferridoxinlike domains (36). Also intriguing is that examination of the published sequence for the hydrogenase structural genes of *B. japonicum* (38) has revealed a Nif promoter consensus sequence −47/−31 bases upstream from the transcriptional start site, although the sequence does not necessarily indicate a functional promoter.

Tn5 mutagenesis was used in a previous study to identify and define *hup*-related loci (14), using clones containing regions of DNA overlapping the region of DNA encoded by pSH22. The results have indicated that a 15.5-kb stretch of DNA, some of which is included on pSH22, contains *hup*-related sequences. The 15.5-kb stretch extends rightwards from the hydrogenase transcriptional start site (Fig. 4) (14, 38, 43). The putative regulatory locus identified here resided outside of that region. Thus, in this study, sequences were defined as *hup* related which have not been so defined previously. The locus presumably missed identification in the studies of Haugland et al. (14) and those following (20, 21, 43), because it falls within a 3-kb gap lacking Tn5 insertions in those studies. Conclusions reached by experiments in which hydrogen uptake activity has been transferred interspecies or intergenera (14, 19–21) are not contradicted by the discovery of this additional gene. In those experiments, the transfers included the putative regulatory segment of DNA described here, since it is adjacent to the hydrogenase structural gene loci.

While the characteristics of mutant SR139 indicated that the locus was also involved in regulation of the Nif phenotype, definitive assignation awaits further characterization. Preliminary evidence showed that cosmid pSH22 could complement both the Nif and Hup functions of SR139 (15),

but those experiments could not be extended to the subclones generated from pSH22. When symbiotic assays were attempted, the plasmids of the transconjugants were lost in the absence of selection pressure. This problem has been described previously for vectors pLAFR1 and pRK290 (4, 15, 19, 25). When other researchers have been able to measure symbiotic nitrogenase activities in plants inoculated by such cosmid-containing bacteria, the levels of activity have been very low, and the level of plasmid retention has also been low (4, 14, 15). The low level of nitrogenase activity inducible in free-living cultures of strain SR and derivatives meant ex planta assays of nitrogenase were not reliable for SR139 and transconjugants. Nor were several attempts to cross the Tn*5*-mutated region of pRY12 into the genome successful. Thus, although available data suggested that the defect of SR139 was in a single locus which regulates both Hup and Nif, only the former assertion could be demonstrated conclusively in these studies. Logically, the two enzymes, although surely possessing some independent mechanisms of regulation, would also be regulated by some common means. However, final proof that a *nif/hup* locus has been identified in SR139 awaits a better means of assaying the nitrogenase-related portion of the mutation.

Whether or not the gene identified is also involved in the regulation of nitrogenase, it was at least demonstrated to be involved in the expression of hydrogenase activity. Thus, a putative regulatory gene was identified and was shown to constitute a new example of a *hup*-related gene of *B. japonicum*; the region of DNA considered to contain *hup*-specific DNA was also extended. Now that the location of the gene on the cloned DNA has been determined, it should be useful in examining the regulation and expression of the hydrogenase, and perhaps also the nitrogenase, enzyme.

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