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Characterization of Three Genomic Loci Encoding *Rhizobium* sp. Strain ORS571 N_2 Fixation Genes

ROBERT G. K. DONALD, DAVID W. NEES, CHRISTOPHER K. RAYMOND, ALBERT I. LOROCH, AND ROBERT A. LUDWIG*

Department of Biology, Thimann Laboratories, University of California, Santa Cruz, California 95064

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Sixty-five independent, N_2 fixation-defective (Nif$^-$) vector insertion (Vi) mutants were selected, cloned, and mapped to the ORS571 genome. The recombinant Nif::Vi plasmids obtained in this way were used as DNA hybridization probes to isolate homologous phages from a genomic library of ORS571 constructed in λEMBL3. Genomic maps were drawn for three ORS571 Nif gene loci. Forty-five Nif::Vi mutants in genomic Nif locus 1 defined two gene clusters separated by 8 kilobase pairs (kb) of DNA. In the first cluster, 36 Nif::Vi mutants mapped to a 7-kb DNA segment that showed DNA homology with *Klebsiella pneumoniae nifHDKE* and encoded at least two Nif operons. In the other cluster, nine Nif::Vi mutants mapped to a 1.5-kb DNA segment that showed homology with *K. pneumoniae* and *Rhizobium meliloti nifA*; this DNA segment encoded a separate Nif operon. Fifteen Nif::Vi mutants mapped to a 3.5-kb DNA segment defined as Nif locus 2 and showed DNA homology with the *R. meliloti* P2 *fixABC* operon. Nif locus 2 carries a second *nifH* (*nifH2*) gene. Four Nif::Vi mutants mapped to a 2-kb DNA segment defined as Nif locus 3 and showed DNA homology with *K. pneumoniae nifB*. DNA from λNif phages comprising all three genomic Nif loci was subcloned in plasmid vectors able to stably replicate in ORS571. These plasmid subclones were introduced into ORS571 strains carrying physically mapped Nif::Vi insertions, and genetic complementations were conducted. With the exception of certain mutants mapping to the *nifDK* genes, all mutants could be complemented to Nif$^+$ when they carried plasmid subclones of defined genomic DNA regions. Conversely, most *nifDK* mutants behaved as pseudodominant alleles.

Rhizobium sp. strain ORS571, uniquely among characterized members of the family *Rhizobiaceae*, conducts N_2 fixation during active bacterial growth (16) as well as during plant nodule symbiosis, in which it is a nonproliferating endosymbiont. ORS571 forms classical, synchronous nodules in roots and in stem lateral root primordia with the host leguminous plant *Sesbania rostrata* (32). As such, ORS571 appears to be a chimeric N_2-fixing bacterium with properties of both orthodox rhizobia and diazotrophic bacteria. Operationally, the chimeric nature of ORS571 N_2 fixation facilitates a genetic analysis of rhizobial N_2 fixation processes because ORS571 N_2 fixation-defective (Nif$^-$) mutants may be directly selected and screened ex planta.

We have recently developed the vector-insertion (Vi) mutagenesis-cloning strategy and have applied it to ORS571 (9). This method allows the direct molecular cloning of any ORS571 genes in which mutants can be selected or screened. Vi mutagenesis-cloning consists of the following protocol. Tn*5* is first introduced into ORS571 on limited-host-range plasmids that cannot replicate in this strain. Stable ORS571 Tn*5* insertions are selected and subsequently screened for Vi mutants. Mutant strains invariably exhibit IS*50*R-mediated, complete plasmid-genome cointegrates in lieu of Tn*5*. Analyses of plasmid-genome cointegrates of many such Vi mutants show that IS*50*R-mediated vector insertions are random, bidirectionally oriented, and stable. Genomic structures of Vi mutants thus exhibit single-copy DNA sequences of the entire incoming plasmid pVP2021 flanked by direct IS*50*R repeats.

For each ORS571 Vi mutant strain of interest, the genomic VP2021 DNA sequences constitute a targeted cloning vector. Total genomic DNA is isolated and digested with *Eco*RI. DNA ligations of genomic *Eco*RI digests are conducted, without adding an exogenous DNA vector, under dilute conditions favoring circularization of all fragments. Ligation mixtures are used to transform *Escherichia coli*. Recombinant plasmids from these *E. coli* transformants may be physically mapped and used as DNA hybridization probes against the ORS571 genomic library to identify recombinant phages carrying corresponding wild-type genes.

In this report, we describe extensive use of the targeted Vi mutagenesis-cloning technique to isolate, characterize, and map three ORS571 Nif gene loci.

MATERIALS AND METHODS

Bacterial strains and plasmids: Vi mutagenesis-cloning. To isolate Vi mutants, *Rhizobium* sp. strain ORS571 was plated with *E. coli* donor strains carrying suicide mutagenesis plasmid pVP2021 (Table 1). ORS571 recipients carrying IS*50*R-mediated plasmid-genome cointegrates were obtained after platings on rich media containing kanamycin and streptomycin as described previously (9). From this collection, candidate strains were screened by replica plating on ORSNif medium, a defined, twofold-diluted M9 minimal medium containing 0.4% D-glucose, 0.4% succinic acid titrated to pH 6.0 with KOH, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 μg of $FeCl_3$ per ml, 1 μg of $NaMoO_4$ per ml, 1 μg of pantothenate per ml, 1 μg of nicotinate per ml, and 0.3 μg of D-biotin per ml, with and without 15 mM $(NH_4)_2SO_4$ added as a nitrogen source. Plates were solidified with 0.85% agarose (Clonetech) and incubated in a growth chamber at 30°C under a continous flow atmosphere of 98% N_2–1% CO_2–1% O_2. ORS571 Nif::Vi mutants were identified by their inability to utilize N_2 as a nitrogen source on solid media (9). Putative

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and Nif-subcloned plasmids

Strains and plasmids	Genotype or phenotype	Reference or source
E. coli		
HB101	Pro Thi Leu *recA hsdS supE4*	
SM10	MM294 [::pRP4ΔTn1 Tc^s] *recA* Tra $(IncP1)^+$ Kan^r	29
Rhizobium spp.		
RS57100	ORS571 wild type	
RS57111	ORS571 Nal^r, Spc^r	
Plasmids		
pVP2021	Vi mutagenesis-cloning vector	9
pRK2013	ColE1::pRK2 Km^r Tra^+	7
pWK220	*K. pneumoniae* $NifQ^+B^+A^+L^+F^+M^+V^+S^+U^+X^+D^+E^+D^+N^+Y^+K^+D^+H^+J^+$	22
pWK23	*K. pneumoniae* $NifQ^+B^+A^+L^+$	22
pUC8*nifL*$^+$	*K. pneumoniae* $NifL^+$	A. Pühler
pSA30	*K. pneumoniae* $NifH^+D^+K^+Y^+$	3
pDC8	*R. meliloti* P2 *fix* operon, $NifA^+$	D. Corbin
ORS571 Nif plasmid DNA subclones		
pLAFRBX	pLAFRB-*Xho*I linker (*Eco*RI-*Bam*HI-*Xho*I-*Bam*HI-*Eco*RI)	6; this study
pNSN11	pLAFRBX Nif locus 1	
pNSN15	pLAFRBX Nif locus 1	
pNSN18	pLAFRBX Nif locus 1	
pRS1	pRK290 Nif locus 1	11
pNSN21	pRK311 Nif locus 2	Ditta et al., in press; this study
pNSN31	pRK293 Nif locus 3	Ditta et al., in press; this study

Nif::Vi mutants strains were retested for growth on ORSNif medium and tested for whole cell nitrogenase activity in ORSNif medium by the acetylene reduction assay, as described previously (8). For Vi cloning, total genomic DNAs from these strains were isolated, purified by cesium chloride-ethidium bromide isopycnic centrifugation, completely digested with *Eco*RI restriction endonuclease, and ligated (without the addition of an exogenous cloning vector) under dilute conditions favoring intramolecular DNA ligations, as described previously (9). Ligation mixtures were used to transform *E. coli* HB101, and tetracycline-resistant transformants were selected, as described previously (9). ORS571 Nif::Vi mutant strains and the recombinant plasmid DNAs derived from them are listed in Table 2.

Construction of the *Rhizobium* sp. strain ORS571 genomic DNA library. A complete library of *Rhizobium* sp. strain ORS571 genomic DNA was prepared in λEMBL3 (14) by standard techniques (14, 20). ORS571 genomic DNA was purified by twice-repeated cesium chloride-ethidium bromide isopycnic centrifugation, partially digested with restriction endonuclease *Mbo*I, and size-fractionated by sedimentation on sucrose gradients (20). λEMBL3 cloning vector DNA was prepared from DNA purified as described above and digested with restriction endonuclease *Bam*HI, and vector arms were resolved after sedimentation on sucrose gradients as described previously (14, 20). DNA ligation of left and right vector arms and ORS571 target DNA was accomplished with purified phage T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, Md.). Ligation mixtures were λ packaged in vitro and plaque amplified by standard techniques (20).

Construction of ORS571 Nif plasmid subclones. Isolated λNif phage DNAs were subcloned in the stable, low-copy-number, broad-host-range plasmid pRK290 and derivative plasmids (6a, 7) (Table 1). These subcloned plasmids were stored as *E. coli* HB101 transformants. The resulting subcloned ORS571 Nif plasmids are also listed in Table 1; subcloned plasmids carrying ORS571 genomic inserts were mapped and diagrammed (see Fig. 2, 3, and 4).

Genetic complementation tests. Plasmid subclones were transferred from *E. coli* to *Rhizobium* sp. strain ORS571 during heterogeneric matings with *E. coli* IncP1-mobilizing strain SM10 (29) or helper plasmid pRK2013 (7) as donors. Genetic complementation tests for a Nif^+ phenotype were conducted in ORS571 Nif::Vi mutant strains carrying defined plasmid subclones, as described in the text.

DNA hybridization reactions. ORS571 bacteroids from *S. rostrata* stem root primordia nodules were prepared with self-generating Percoll density gradients (24), and bacteroid DNA was extracted by a sarcosyl detergent wash-osmotic shock lysis procedure (27). ORS571 genomic, bacteroid, and plasmid DNA isolations and hybridizations were performed as described previously (9), but with the following modification: 50 μg of heparin per ml was included in DNA hybridization reactions (30). DNA hybridizations with heterologous DNA probes were conducted at 37°C for 48 h, and nitrocellulose membranes were washed in 5× SSPE (5) for 2 h at 42°C. Restriction enconuclease-digested DNA fragments were separated and purified by electroelution (20) or by DEAE membrane capture (Schleicher & Schuell, Inc., Keene, N.H.). For use as DNA hybridization probes, heat-denatured DNA fragments were randomly radiolabeled with [α-^{32}P]dCTP with a purified *E. coli* DNA polymerase I Klenow fragment (New England Biolabs, Inc., Beverly, Mass.), using random oligodeoxynucleotides as primers (12). Intact plasmid and phage DNAs were radiolabeled with [α-^{32}P]dCTP by a previously described nick-translation procedure (5).

Preparation of *Klebsiella pneumoniae* and *Rhizobium meliloti* DNA fragments for use as heterologous DNA hybridization probes. A *K. pneumoniae* M5A1 DNA fragment carrying *nifHDK* genes was prepared by *Eco*RI digestion of pSA30 (3); a 3.6-kilobase-pair (kb) DNA fragment carrying the *nifLA* genes and the proximal end of the *nifB* gene was

TABLE 2. Phenotypes, Vi plasmids from, and Nif$^+$ complementation tests with ORS571 Nif::Vi mutants

Strain	Phenotype			Vi plasmid	Nif$^+$ complementation by[a]:					
	Nif (g)[b]	Nif (r)[c]	Fix[d]		pNSN15	pNSN11	pRS1	pNSN18	pNSN21	pNSN31
60109	−	−	−	pDRN109	−	+	−	−	·	·
60215	−	−	−	pDRN215	−	+	−	−	·	·
60224	−	−	−	pDRN224	−	+	−	−	·	·
60240	−	−	−	pDRN240	−	+	−	−	·	·
60004	−	−	−	pDRN4	−	−	+	−	−	·
60008	−	−	−	pDRN8	−	−	−	−	·	·
60009	−	−	−	pDRN9	−	−	+	−	−	·
60016	−	−	−	pDRN16[e]	−	−	+	−	−	−
60029	−	−	−	pDRN29	−	−	−	−	−	·
60035	−	−	−	pDRN35	−	−	−	−	·	·
60041	−	−	−	pDRN41	−	−	−	−	·	·
60047	−	−	−	pDRN47	−	−	−	−	·	·
60055	−	−	−	pDRN55	−	−	+	−	−	·
60057	−	−	−	pDRN57	−	−	−	−	·	·
60070	−	−	−	pDRN70[f]	−	−	+	−	·	·
60072	−	−	−	pDRN72	−	−	+	−	·	·
60102	−	−	−	pDRN102	−	−	−	−	·	·
60103	−	−	−	pDRN103	−	−	+	−	·	·
60104	−	−	−	pDRN104	−	−	+	−	·	·
60108	−	−	−	pDRN108	−	−	−	−	·	·
60110	−	−	−	pDRN110	−	−	+	−	·	·
60112	−	−	−	pDRN112	−	−	−	−	·	·
60116	−	−	−	pDRN116	−	−	+	−	·	·
60202	−	−	−	pDRN202	−	−	−	−	−	·
60203	−	−	−	pDRN203	−	−	−	−	·	·
60205	−	−	−	pDRN205	−	−	−	−	·	·
60206	−	−	−	pDRN206	−	−	−	−	·	·
60208	−	−	−	pDRN208	−	−	−	−	·	·
60209	−	−	−	pDRN209	−	−	+	−	·	·
60210	−	−	−	pDRN210	−	−	−	−	·	·
60228	−	−	−	pDRN228	−	−	−	−	·	·
60230	−	−	−	pDRN230	−	−	−	−	·	·
60231	−	−	−	pDRN231	−	−	+	−	·	·
60236	−	−	−	pDRN236	−	−	−	−	·	·
60237	−	−	−	pDRN237	−	−	−	−	·	·
60239	−	−	−	pDRN239	−	−	+	−	·	·
60244	−	−	−	pDRN244	−	−	−	−	·	·
60034	−	−	−	pDRN34	+	−	−	−	·	·
60051	−	−	−	pDRN51	+	−	−	−	·	·
60105	−	−	−	pDRN105	+	−	−	−	·	·
60107	−	−	−	pDRN107	+	−	−	−	·	·
60201	−	−	−	pDRN201	+	−	−	−	·	·
60214	−	−	−	pDRN214	+	−	−	−	·	·
60225	−	−	−	pDRN225	+	−	−	−	·	·
60226	−	−	−	pDRN226	+	−	−	−	·	·
60005	−	+/−	−	pDRN5	−	−	−	−	+	·
60017	−	+/−	−	pDRN17	−	−	−	−	+	·
60019	−	+/−	−	pDRN19	−	−	−	−	+	·
60024	−	+/−	−	pDRN24	−	−	−	−	+	−
60031	−	+/−	−	pDRN31	−	−	−	−	+	·
60062	−	+/−	−	pDRN62	−	−	−	−	+	·
60063	−	+/−	−	pDRN63	−	−	−	−	+	·
60101	−	+/−	−	pDRN101	−	−	−	−	+	·
60113	−	+/−	−	pDRN113	−	−	−	−	+	·
60114	−	+/−	−	pDRN114	−	−	−	−	+	·
60117	−	+/−	−	pDRN117	−	−	−	−	+	·
60118	−	+/−	−	pDRN118	−	−	−	−	+	·
60207	−	+/−	−	pDRN207	−	−	−	−	+	·
60218	−	+/−	−	pDRN218	−	−	−	−	+	·
60222	−	+/−	−	pDRN222	−	−	−	−	+	·
60229	−	+/−	−	pDRN229	−	−	−	−	+	·
60028	−	−	−	pDRN28	−	−	−	−	·	+
60213	−	−	−	pDRN213	−	−	−	−	·	+
60216	−	−	−	pDRN216	−	−	−	−	·	+
60220	−	−	−	pDRN220	−	−	−	−	·	+

[a] Results of complementation tests are abbreviated as follows: +, positive complementation; −, negative complementation; ·, not tested.
[b] N_2-dependent growth (g) assay on ORSNif solid medium.
[c] Acetylene reduction (r) activity in free-living culture.
[d] Symbiotic acetylene reduction activity in planta.
[e] Referred to previously (9) as pDRN161.
[f] Referred to previously (9) as pDRN701.

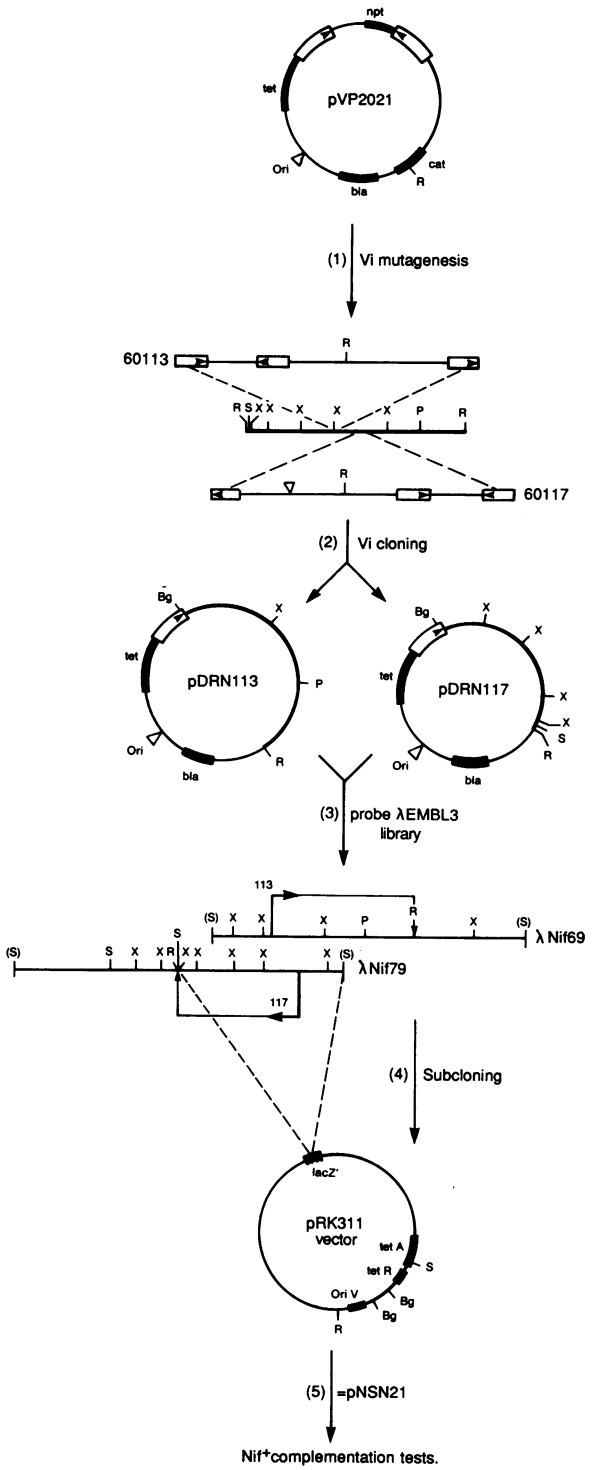

FIG. 1. Schematic representation of the strategy used in the targeted Vi mutagenesis-cloning of ORS571 *nif* genes. ORS571 Nif locus 2 is presented as an example. Step 1: Vi mutagenesis. Plasmid pVP2021 was transferred to ORS571 and yielded Nif::VP2021 insertion mutants in the form of IS*50*$_R$-mediated plasmid-genome cointegrates (9). Step 2: Cloning of Vi mutants, using targeted VP2021 sequences as endogenous cloning vector. In Nif::Vi mutants 60113 and 60117, genomic DNA sequences flanking insertion points yielded recombinant plasmids pDRN113 and pDRN117, respectively, after *Eco*RI digestion of total genomic DNAs, intramolecular DNA ligations, and transformations of *E. coli* strain HB101. Recombinant plasmids derived from genomic insertions in the same genomic *Eco*RI DNA fragment but in opposite orientations exhibited overlapping genomic DNA sequences and allowed the target genomic *Eco*RI DNA fragment to be physically mapped. Step 3: Recombinant plasmids were employed as DNA hybridization probes to obtain recombinant phages that carried wild-type *nif* gene regions from the ORS571 λEMBL3 genomic library. Two genomically overlapping, recombinant phages, λNif69 and λNif79, which together carried the DNA inserts comprising ORS571 Nif locus 2, were identified by DNA homology with recombinant plasmid DNA inserts from pDRN113 and pDRN117. The genomic DNA inserts of plasmids pDRN113 and pDRN117 are, in turn, represented schematically on the recombinant phage DNA insert maps. Step 4: Subcloning of recombinant phage DNA inserts into broad host-range pRK290-derived plasmids (7; Ditta et al., in press). Step 5: Nif$^+$ complementation analyses of Nif::Vi mutants that carry plasmid subclones of the corresponding wild-type regions. Plasmid pNSN21, containing DNA subclones from λNif79, complemented to Nif$^+$ mutants 60113, and 60117 and all other Nif locus 2 mutants (see text). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I. EMBL3 *Sal*I sites are represented in parentheses.

prepared by *Sma*I digestion of pWK23 (22); a 1.7-kb DNA fragment carrying most (the distal end) of the nifB gene was prepared by *Hin*dIII-*Sal*I double digestion of pWK23 (22); a 2.1-kb DNA fragment carrying the *nifNE* genes was prepared by *Eco*RI digestion of pWK220 (22). A 4.5-kb DNA fragment from the *R. meliloti* 102F34 P2 *fixABC* operon (2, 4, 31) was prepared by *Hin*dIII digestion of pDC8. A 1.6-kb DNA fragment carrying *R. meliloti nifA* was prepared by *Bam*HI digestion of pDC8 (2, 4, 31); a DNA fragment carrying *R. meliloti nifB* (2) was prepared by *Bam*HI-*Bgl*II double digestion of pDC8 (4).

RESULTS

Isolation of ORS571 Nif::Vi mutants. Approximately 100 independent *Rhizobium* sp. strain ORS571 Nif::Vi mutants were identified by their defective growth on solid medium for which atmospheric N_2 was the nitrogen source. Nif$^-$ phenotypes were verified by nitrogenase assays (see above). Of these, 65 independent Nif::Vi mutants were identified that (i) were unambiguously unable to grow on ORSNif solid medium and (ii) displayed less than 1% of the ORS571 wild-type nitrogenase activity in acetylene reduction assays (see above). These tight Nif$^-$ mutants were chosen for Vi cloning. The complete experimental strategy for the analysis of these mutants is schematically presented in Fig. 1.

Targeted Vi cloning of mutagenized ORS571 Nif genes. Purified total genomic DNAs from these 65 ORS571 Nif::Vi mutant strains were digested to completion with excess *Eco*RI endonuclease. DNA restriction fragments were circularized by DNA ligation reactions conducted under dilute conditions to kinetically favor intramolecular ligations (9). DNA ligation mixtures were used to transform *E. coli* HB101, Tc^r colonies were selected, and these bacteria carried recombinant plasmids (9). When the recombinant DNA was derived from a given ORS571 Nif::Vi strain, only a single recombinant plasmid species was isolated among many *E. coli* Tc^r transformants. In previously reported genomic DNA hybridization experiments, 12 Vi mutant strains tested have been shown to carry single-copy Vi insertions (9). A complete list of recombinant plasmids obtained from parental mutant strains is given in Table 2.

Isolation of ORS571 wild-type Nif DNA gene clusters from the ORS571 λEMBL3 genomic library. Two strategies were

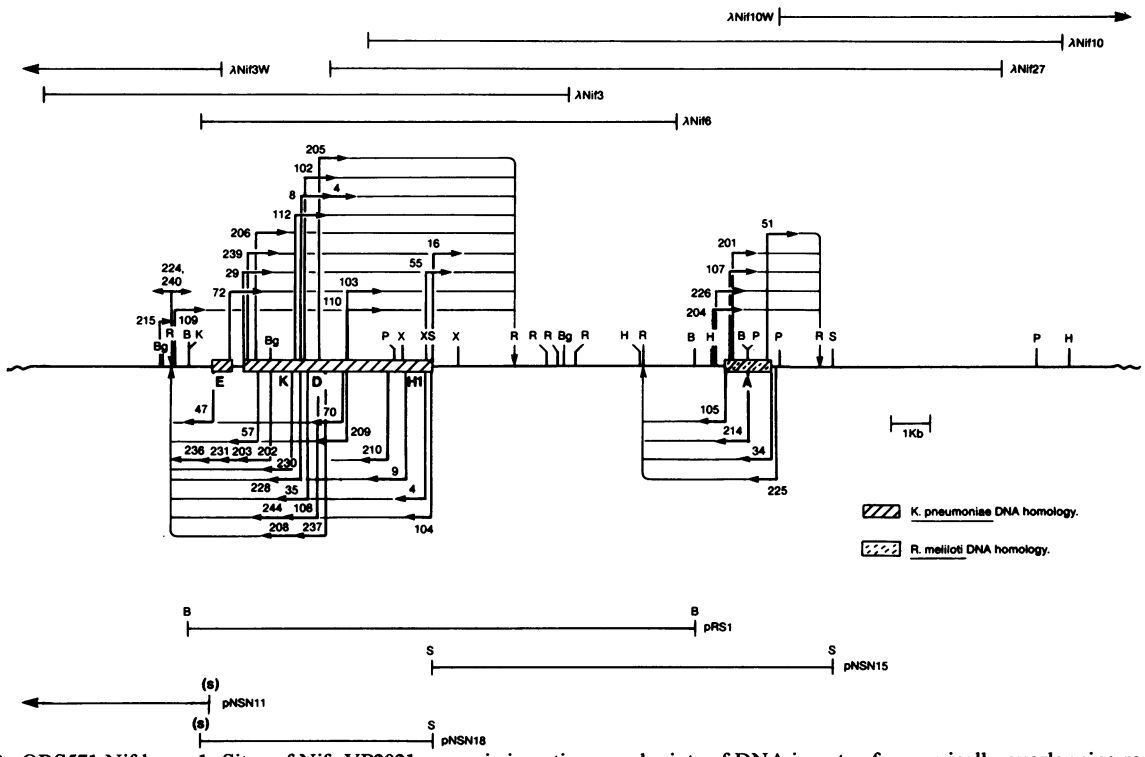

FIG. 2. ORS571 Nif locus 1. Sites of Nif::VP2021 genomic insertions, endpoints of DNA inserts of genomically overlapping recombinant phages and plasmid subclones, and regions of heterologous DNA homology are presented. Vi mutants are represented by identifying strain numbers. Extents of genomic DNA inserts in derived recombinant plasmids are indicated (see Fig. 1) as distances to adjacent genomic *Eco*RI sites. DNA inserts of plasmid subclones pNSN11, pRS1, pNSN15, and pNSN18 that were derived from Nif locus 1 are mapped. Abbreviations are as defined in the legend to Fig. 1.

employed to obtain ORS571 *nif* gene DNA fragments from the genomic λEMBL3 library (see above). In the first strategy, plasmid pSA30 carrying the *K. pneumoniae nifHDK* genes (3) was used as a DNA hybridization probe. Recombinant phages λNif3, λNif6, λNif10, and λNif27 possess DNA inserts that together comprise ORS571 Nif locus 1 (Fig. 2). In the second strategy, recombinant plasmids derived from Vi mutants were used as DNA hybridization probes (Fig. 1). Recombinant plasmids derived from Nif::Vi mutants carry a variable amount of ORS571 Nif DNA sequences that map from the point of the genomic insertion to the immediately adjacent genomic *Eco*RI site (Fig. 1). By these two experimental strategies three physically unlinked genomic ORS571 Nif gene loci were successfully identified.

To illustrate the second scheme (Fig. 1), DNA inserts of recombinant plasmids pDRN113 and pDRN117 (Table 2) were used as hybridization probes in a series of λ plaque DNA hybridization screenings (5, 20). DNA inserts from

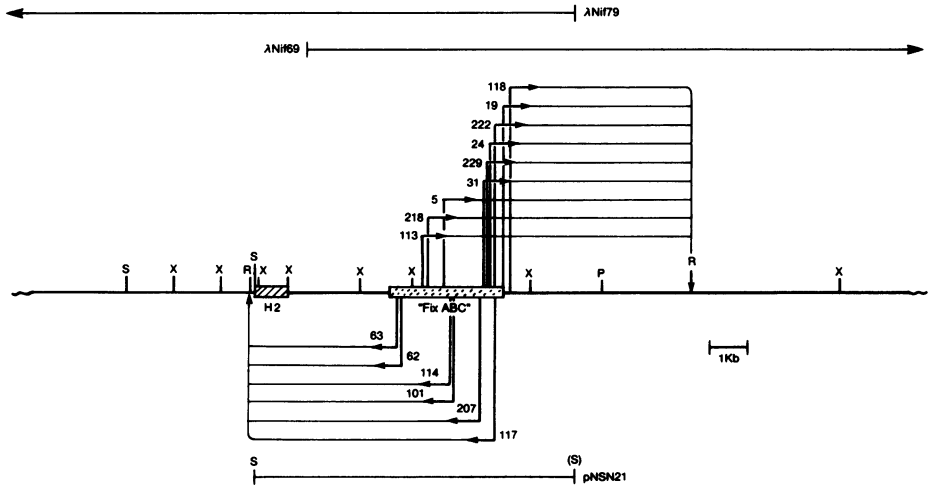

FIG. 3. ORS571 Nif locus 2. (see Fig. 2). Abbreviations are as defined in the legend to Fig. 1.

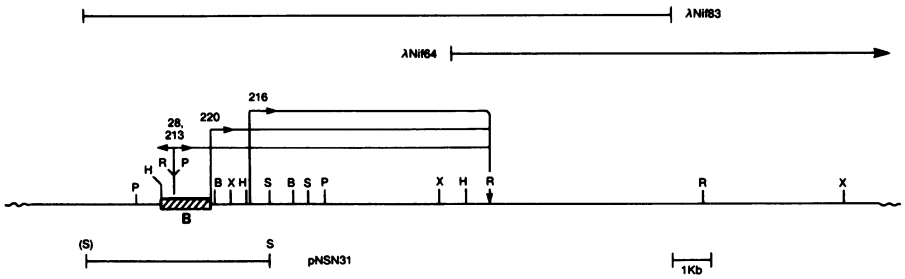

FIG. 4. ORS571 Nif locus 3. (see Fig. 2). Abbreviations are as defined in the legend to Fig. 1.

pDRN113 and pDRN117 were prepared by double digestion with *Eco*RI and *Bgl*II endonucleases. Recombinant plasmids invariably carried a single genomic hybrid *Eco*RI site at which ligation had occurred. Because *Bgl*II sites occur only 50 nucleotides in from the external boundaries of IS*50* elements, specific ORS571 genomic fragments were generated that carried, in addition, 50 base pairs of IS*50* DNA (Fig. 1). These DNA fragments were purified by agarose gel electrophoresis, subsequently radiolabeled, and used in DNA hybridization experiments (see above). Two strongly hybridizing recombinant phages, λNif69 and λNif79, were identified and characterized. Both phages strongly cross-hybridized, contained DNA inserts homologous to both pDRN113 and pDRN117, and collectively spanned 35 kb of genomic ORS571 DNA. Phages λNif69 and λNif79 insert DNAs comprised ORS571 Nif locus 2 (Fig. 3).

Recombinant plasmid pDRN220, derived from strain 60220, contained a 7.5-kb DNA insert and showed no DNA homology to the recombinant phage DNA inserts comprising ORS571 Nif loci 1 and 2. Therefore, the pDRN220 DNA insert was used as a DNA hybridization probe, and recombinant phage λNif83, the insert DNA of which constitutes ORS571 Nif locus 3, was obtained (Fig. 4).

Genomic mapping of ORS571 Nif::Vi genomic insertion sites: Nif locus 1. Of 65 recombinant DNA plasmids derived from the independently isolated ORS571 Nif::Vi strains, 31 showed recombinant DNA insert homology, localized to a 5.7-kb DNA region, with the λNif6 phage (Fig. 2). For these DNA hybridization experiments, the two *Sal*I recombinant DNA fragments from λNif6 (9) were isolated, radiolabeled, and independently used as DNA hybridization probes (see above). Restriction endonuclease mapping analyses allowed and DNA hybridization confirmed the precise locations of the original ORS571 genomic insertion sites for these 31 Nif::Vi mutant strains (Fig. 2). In heterogeneric DNA hybridization experiments, λNif6 recombinant insert DNA also showed homology to *K. pneumoniae nifHDK* genes that encode the structural proteins for components 1 and 2 of nitrogenase.

Overlapping genomic clones λNif3w and λNif10w from ORS571 Nif locus 1 were identified by genomic walking DNA hybridization experiments, using recombinant DNA inserts from λNif3 and λNif10 phages, respectively, as DNA hybridization probes. When the 55-kb DNA extended physical map of Nif locus 1 was constructed, an additional 14 ORS571 Nif::Vi mutants could be mapped precisely. Plasmids pDRN109, pDRN215, pDRN240, and pDRN224, with genomic inserts of 9, 0.3, 0.1, and 0.1 kb of DNA, respectively, showed DNA homology to the λNif3 DNA insert. Plasmid pDRN109 showed additional DNA homology to both *Sal*I DNA fragments of λNif6 (9) and displayed DNA insert fragments (*Eco*RI-*Xho*I, *Eco*RI-*Bgl*III) common to λNif6 and plasmids pDRN72, pDRN29, and pDRN239 (Fig. 2). Thus, Nif::Vi mutants in strains 60109, 60215, 60224, and 60240 mapped near an *Eco*RI site located 2 kb distal to the *nifH1 nifD nifK* region (11).

Restriction endonuclease mapping analyses of recombinant plasmids obtained from strains 60034, 60051, 60105, 60107, 60201, 60204, 60214, 60225, and 60226 identified DNA inserts that mapped within a 4.7-kb genomic *Eco*RI DNA fragment. The recombinant plasmids derived from these nine strains showed DNA homology with λNif10, a Nif locus 1 recombinant phage (Fig. 1). Several of these plasmids also showed homology to heterologous *K. pneumoniae* and *R. meliloti nifA*-containing DNA hybridization probes (see above). Although the *K. pneumoniae* hybridization probe is multiallelic (3, 30), the *R. meliloti* probe carries only *nifA* sequences (2). Therefore, these nine mutants reside in an operon encoding *nifA*. The *nifA* gene product in *K. pneumoniae* acts as the transcriptional activator of its *nif* operons (21); the *R. meliloti nifA* gene may regulate its *nif* operons similarly (2, 31). Heterologous *nifA* DNA homology was localized to the 1.8-kb genomic ORS571 DNA segment spanned by these nine Nif::Vi mutants. The extent of *K. pneumoniae* and *R. meliloti nifA* DNA homology was estimated as 1.4 kb of DNA. In addition to the 0.9-kb *Pst*I DNA fragment, the *R. meliloti nifA* probe hybridized to an adjacent *Pst*I DNA fragment (*Bgl*III-*Pst*I DNA fragment of pDRN226); the *K. pneumoniae nifA* probe yielded only weak hybridization with this same *Pst*I DNA fragment. In contrast, plasmids pDRN51 and pDRN105, derived from Nif::Vi mutant strains 60051 and 60105, showed no heterologous *nifA* homology. The genomic map positions and orientations of these Nif::Vi mutants thus delineated the extent of heterologous *nifA* DNA homology (Fig. 2). No ORS571 DNA homology was detected when isolated *K. pneumoniae nifL* DNA fragments were used to probe (i) ORS571 total genomic DNA and (ii) recombinant phages. *K. pneumoniae nifL* encodes a Nif regulatory protein that is sensitive to the cytoplasmic membrane redox potential; the *nifL* gene is located in the same operon but distal to *nifA* (21).

The extent of ORS571 DNA homology to plasmid pSA30, which carries the *K. pneumoniae nifHDK* genes, was determined by heterologous DNA hybridizations with recombinant plasmids derived from Nif::Vi mutants localized to Nif locus 1. Results agreed with those determined previously (11); the homologous ORS571 genomic region was slightly extended because both plasmids pDRN55 and pDRN57 carried DNA fragments with detectable homology (Fig. 2). The physical locations of ORS571 *nifH1*, *nifD*, and *nifK* genes within this 5-kb DNA region were approximated by DNA hybridizations with a physically ordered array of recombinant plasmids derived from Nif::Vi mutant strains

and the staggered array of λNif3, λNif10, and λNif27 (Fig. 2); pSA30 DNA fragments were used as DNA hybridization probes with these recombinant phage DNA inserts. In subsequent genome mapping experiments, only a 0.7-kb *Xho*I genomic DNA fragment showed homology to the 0.9-kb *Eco*RI-*Bgl*II *nifH*-containing pSA30 DNA fragment. λNif3 and λNif27, but not λNif10, DNA inserts showed homology to the 1.65-kb *Bam*HI-*Hin*dIII *nifD*-containing DNA fragment; likewise λNif3, but neither λNif10 nor λNif27, showed homology to the 1.6-kb *Hin*dIII-*Sal*I *nifK*-containing DNA fragment (Fig. 2). A total of 29 ORS571 Nif::Vi mutants, mapped to Nif locus 1, lay in the region displaying DNA homology with pSA30. Of these, 5 were mapped to the ORS571 *nifH1* gene, 25 to the *nifD* gene, and 8 to the *nifK* gene.

Similarly, DNA homology with *K. pneumoniae nifE* was detected and mapped immediately distal to the ORS571 *nifK* gene. An *Eco*RI DNA fragment of plasmid pWK220 containing *K. pneumoniae nifNE* was isolated and used as a DNA hybridization probe in these experiments. DNA homology to λNif3, λNif6, and plasmid pDRN57 was observed. Other work has further delimited the ORS571 DNA homology to include *nifE* and exclude *nifN* (21a). Because no DNA homology was detected with plasmid pDRN47 or pDRN72, the region of *K. pneumoniae nifE* homology was limited to 0.4 kb between the sites of the insertions in strains 60047 and 60072 (Fig. 2). In *K. pneumoniae*, the *nifE* product is necessary for the maturation of nitrogenase iron-molybdenum component 1, on which the iron-molybdenum cofactor is constructed (25).

No phenotypic differences among the 45 ORS571 Nif locus 1 mutants were discerned. All displayed Nif$^-$ phenotypes when plated on ORSNif solid medium (see above), all were without detectable acetylene reduction activity, and all displayed Nod$^+$ and Fix$^-$ phenotypes in nodulation tests with *S. rostrata*.

Genomic mapping of ORS571 Nif::Vi genomic insertion sites: Nif locus 2. Fifteen independently isolated ORS571 Nif::Vi mutants were mapped to an 11.7-kb *Eco*RI DNA fragment comprising Nif locus 2 (Fig. 3). Genomic walking DNA hybridization experiments with phage from the genomic ORS571 library were unable to link Nif loci 1 and 2. It was concluded that Nif loci 1 and 2 are more than 30 kb of DNA apart. The 15 Nif locus 2 mutants could be phenotypically distinguished from those of Nif locus 1. Whereas these Nif locus 2 mutants displayed a Nif$^-$ phenotype when tested for growth on ORSNif solid medium and yielded Fix$^-$ *S. rostrata* nodules, all exhibited detectable amounts of nitrogenase activity, when measured by acetylene reduction (see above), that ranged from 1 to 5% of the wild-type activity.

These 15 Nif locus 2 mutants were clustered in a 3.5-kb DNA region that showed DNA homology with a DNA fragment carrying the *R. meliloti* P2 *fixABC* operon (2, 4, 31; Table 1). The extent of ORS571 DNA homologous to the P2 operon was found to be delimited by recombinant plasmids pDRN19 and pDRN118 (Table 2 and Fig. 3). For ORS571 DNA fragments generated by double digestion of recombinant plasmids with *Bgl*II and *Xho*I, while pDRN19 and pDRN118 showed no detectable homology with a *R. meliloti* and P2 *fixABC* operon DNA hybridization probe, neighboring insertion-derived plasmid pDRN222 and pDRN63 DNA fragments did show homology (Table 2 and Fig. 3).

DNA cross-hybridization experiments between Nif locus 1 and Nif locus 2 recombinant plasmids and phages revealed ORS571 genomic *nifH* duplication. Physically, the genomic duplication consisted of an ostensibly identical 0.7-kb *Xho*I DNA fragment (that may extend an additional 0.1 kb to a common, immediately adjacent *Sal*I site). The duplicated *Xho*I DNA fragments also strongly hybridized with the 0.9-kb *Eco*RI-*Bgl*II pSA30 *K. pneumoniae nifH* DNA fragment. To more precisely delimit the ORS571 Nif locus 1 and Nif locus 2 DNA cross-homology, and to determine the relative orientations of the duplicated *nifH* genes, further DNA cross-hybridization experiments were conducted. A 1.9-kb *Xho*I DNA fragment, mapping adjacent to *nifH2* and between *nifH2* and the region of *R. meliloti* P2 *fixABC* operon homology, showed DNA homology with the small *Xho*I-*Pst*I DNA fragment from *nifH1* (Fig. 2). Therefore, the ORS571 DNA cross-homology extended beyond the duplicated 0.7-kb *Xho*I DNA fragments but not by more than 0.2 kb of DNA. This oriented the *nifH1* gene from right to left and the *nifH2* gene from left to right (Fig. 2 and 3), although it did not establish absolute orientations for these genes.

Recombinant plasmid pDRN16, derived from Nif::Vi mutant strain 60016, the insertion of which maps to the right of *nifH1* (Fig. 2), exhibited no detectable DNA homology with the duplicated Nif locus 2 0.7-kb *Xho*I DNA fragment; but it did show DNA cross-homology to DNA sequences left of *nifH2* (Fig. 3). Therefore, *nifH2* DNA cross-homology extends to the Nif locus 2 *Eco*RI site that maps 0.2 kb of DNA left of the 0.7-kb *Xho*I DNA fragment (Fig. 3). If *nifH1* is transcribed from right to left (Fig. 2), then *nifH2* is transcribed from left to right (Fig. 3).

Genomic mapping of ORS571 Nif::Vi genomic insertion sites: Nif locus 3. Four independently isolated ORS571 Nif::Vi mutants were mapped to a genomic DNA fragment comprising Nif locus 3 (Fig. 4). Plasmid pDRN220 contained a 7.5-kb recombinant DNA insert and showed no DNA homology to the sets of recombinant phages comprising Nif loci 1 and 2. The pDRN220 recombinant DNA insert was used as a hybridization probe to obtain phages λNif83 and λNif64 from the ORS571 genomic library (Fig. 4). In addition to pDRN220, plasmids pDRN28, pDRN213, and pDRN216 all showed DNA hybridization with λNif83 (Table 2 and Fig. 4). In genomic walking experiments, Nif locus 3 proved to be unlinked to Nif loci 1 and 2. DNA from Nif locus 3 was found to hybridize with a *K. pneumoniae nifBQ* DNA probe (see above). Hybridization was also observed with a *R. meliloti nifB* DNA probe (see above) and confirmed the existence of a *nifB* gene in Nif locus 3. While recombinant plasmids pDRN28 and pDRN213 contained DNA fragments that exhibited *nifB* homology, plasmid pDRN216 did not. This delimited the extent of *nifB* homology (Fig. 4). The four ORS571 Nif locus 3 mutants conferred a Nif$^-$ phenotype indistinguishable from that of the Nif locus 1 mutants.

ORS571 genomic DNA hybridizations. ORS571 contains only two *nifH* genes and single *nifD* and *nifK* genes. When ORS571 total genomic DNA restriction endonuclease digests were probed with the *K. pneumoniae nifH*-specific pSA30 DNA fragment, only ORS571 DNA fragments carrying the *nifH1* and *nifH2* genes exhibited DNA hybridization. Specifically, for the case of ORS571 *Eco*RI-digested total genomic DNA, DNA fragments of 9 and 11.5 kb, which were assigned to Nif loci 1 and 2 (Fig. 2 and 3), respectively, showed hybridization with pSA30 DNA. Corroborating results were obtained when *Bam*HI, *Bgl*II, *Hin*dIII, *Pst*I, and *Sal*I endonucleases were used to prepare genomic DNA digests for *nifHDK* DNA homology experiments.

ORS571 Nif DNA sequences from nodule bacteroids show no genomic rearrangements. Genomic rearrangement of the cyanobacterium *Anabaena* sp. strain 7120 *nifHDK* genes occurs during the course of heterocyst development (17). To

test whether ORS571 genomic *nif* gene rearrangements occur during the development of *S. rostrata* nodules, mature ORS571 bacteroids were isolated from nodules, and total bacteroid DNA was isolated and purified (see above). Total ORS571 bacterial and bacteroid DNAs, digested with *Bam*HI, *Bgl*II, *Hin*dIII, *Pst*I, or *Sal*I, were compared in genomic hybridization experiments. When the two *Sal*I DNA insert fragments of λNif6 corresponding to *nifH1*, *nifD*, *nifK*, and the rightward genomic region (Fig. 2) were used as DNA hybridization probes, no differences in DNA fragment hybridization patterns between bacterial and bacteroid genomic DNAs were evident. It was concluded that rearrangements of ORS571 Nif loci 1 and 2 sequences do not accompany bacteroid development.

Nif$^+$ complementation tests. ORS571 genomic DNA from phages comprising Nif loci 1, 2, and 3 subcloned into stable, broad-host-range plasmid pRK290 and pRK290 derivatives (6a, 7; see above). Resulting ORS571 recombinant Nif plasmids (pNSN series plasmids) were maintained as *E. coli* transformants (Table 2). pNSN series plasmids were transferred to ORS571 Nif::Vi mutants during heterogeneric bacterial conjugations (see above). Various ORS571 Nif::Vi strains carrying pNSN plasmids were subjected to Nif$^+$ complementation tests (see above). Because genomic VP2021 insertions are strongly operon polar, assignable complementation groups in these tests comprise operons and not cistrons. Nif$^+$ complementation tests identified three operons in Nif locus 1 (Table 2). Plasmid pRS1 (Tables 1 and 2) complemented Nif::Vi mutants 60004, 60009, 60016, 60055, 60070, 60072, 60103, 60104, 60110, 60209, 60231, and 60239 (Table 2 and Fig. 2). While all *nifH1*::Vi mutants were reproducibly complemented by plasmid pRS1, most mutants mapping in the *nifD-nifK* region failed to show complementation (Table 2). Likewise, these same noncomplementing mutants, carrying pRS1, failed to show detectable nitrogenase activity, measured as acetylene reduction activity (see above). Plasmid pNSN18, which carries ORS571 *nifH1*, *nifD*, and *nifK* genetic information but lacks DNA sequences at the right (probably proximal) end of *nifH1* (Fig. 2) failed to complement all mutants mapped to this region (Table 2). From these results, it can be determined that pNSN18 cannot carry a promoter for any of these genes.

All Nif::Vi mutants that mapped to the *nifDK* region at least exhibited Nif$^+$ marker rescue in the presence of pNSN18 or pRS1. Tested mutants, carrying either plasmid, were plated on ORSNif medium. Al low frequencies (1 in 10^4 plated cells), Nif$^+$ colonies appeared (Table 2). All Nif$^+$ derivative strains remained stably Nif$^+$ in the absence of selection ($<10^{-4}$ Nif$^-$ mutants). Moreover, most but not all Nif$^+$ derivatives lost both the *tet* allele of pNSN18 or pRS1 and the *tet* and *npt* alleles of the VP2021 (9) insertion mutagen. Thus, in most cases, a double-crossover recombination event and subsequent segregation recreated wild-type Nif$^+$ strains during marker rescue.

Also in Nif locus 1, Nif::Vi mutants in a group comprising strains 60109, 60215, 60240, and 60224 were complemented by plasmid pNSN11 (Table 2 and Fig. 2) which contains as genomic insert a 12-kb DNA fragment of λNif3w. Thus, a discrete Nif operon maps distal to the *nifH1 nifD nifK* region.

The nine *nifA*::Vi mutants 60204, 60226, 60107, 60201, 60051, 60105, 60214, 60034, and 60025 were complemented to Nif$^+$ by pNSN15 (Table 2 and Fig. 2). Thus, *nifA* is part of yet another operon in Nif locus 1.

The 15 mutants of Nif locus 2 were complemented to Nif$^+$ by pNSN21 (Table 2 and Fig. 3). Interestingly, Nif$^-$ *nifH2*::Vi mutants were not obtained. Although this constitutes negative evidence, because all other ORS571 *nif* genes have been targeted as multiple, independent Vi mutants, the failure to observe Nif$^-$ *nifH2*::Vi mutants suggests that (i) *nifH2* is dispensable and (ii) the *nif* genes homologous to the *R. meliloti P2 fixABC* operon are encoded in an operon separate from that encoding *nifH2*.

The four Nif locus 3 Nif::Vi mutants were complemented by the corresponding genomic subclone pNSN31 (Table 2 and Fig. 4). Nif gene(s) in Nif locus 3 constitute a single operon.

Noncomplementing Vi mutants localized to the ORS571 *nifDK* genes behave as pseudodominant alleles. The failure of most *nifDK* Vi mutants to exhibit Nif$^+$ complementation might have resulted from additional mutations in these strains. To test this possibility, representative Vi mutants carrying pRS1 were allowed to form homogenotes, and the mutant plasmids were transferred to strain 57111 (Table 1; wild-type Nif$^+$ background) by selecting for Km^r Sm^r derivatives that remained Nal^r Spc^r. Mutant pRS1 plasmids were mobilized during triparental matings consisting of (i) *E. coli* HB101 carrying the Tra$^+$, limited-host-range plasmid pRK2013 (Table 1), (ii) a selected ORS571 nif::Vi mutant strain, and (iii) the recipient strain 57111. These partial Nif merodiploid strains (mutant plasmid, wild-type genome) were then tested for N_2 fixation properties. Four representative *nifDK* Vi mutants, strains 60009, 60041, 60070, and 60237, were selected for this analysis. It was assumed that these strains would create pRS1 homogenotes at detectable frequencies.

The Nif merodiploid, 57111 derivative strains (mutant plasmid, wild-type genome) fell into two groups when tested for nitrogenase activity. Nif merodiploid strains carrying pRS1Nif::Vi mutations originating from two genomic Nif::Vi mutants complemented by pRS1 (strains 60009 and 60070) exhibited 10 to 20% of the wild-type acetylene reduction activity levels, which are results similar to those of merodiploid strains constructed for the complementation tests (mutant genome, wild-type plasmid). However, 57111-derived, Nif merodiploid strains that carry pRS1nif::Vi mutations originating from two genomic nif::Vi mutants not complemented by pRS1 (strains 60041 and 60237) exhibited essentially no nitrogenase activity (0.6 to 1.7% of the wild-type acetylene reduction rates).

Growth tests with solid ORSNif medium corroborated these results. Strain 57111-derived, Nif merodiploid strains carrying pRS1nif::Vi mutants derived from strains 60009 and 60070 were Nif$^+$ in growth tests; Nif merodipoid strains derived from 60041 and 60237 were Nif$^-$. It was concluded that the failure of certain mutants (strains 60041 and 60237) in the *nifDK* region to exhibit Nif$^+$ complementation with pRS1, indeed, results from the ascribed mutations and that these mutants behave as pseudodominant alleles.

DISCUSSION

The 65 genomically mapped Nif::Vi mutants should have targeted most *Rhizobium* sp. strain ORS571 N_2 fixation genes, unless specialized symbiotic N_2 fixation functions are required. From inspection of the maps (Fig. 2, 3, and 4), the Vi mutagenesis protocol appears to be unbiased and results in random mutants. These maps show an average mutational frequency of one Vi insertion per 250 base pairs of Nif DNA sequences, in which a Nif DNA sequence is defined as DNA spanning any given cluster of Nif$^-$ insertions.

Because *nifH1*::Vi mutants are Nif$^-$, they cannot be intragenomically complemented by *nifH2*$^+$. Following this

logic, because *nifH1*::Vi mutations may be inferred to be polar on at least one distal gene, *nifH1* must be only part of an operon. Inexplicably, Nif$^+$ complementation tests for mutants that map in the *nifDK* region do not yield consistent results. On the one hand, some scattered mutants that map to the *nifDK* region are complemented to Nif$^+$ when carrying pRS1. This suggests that the *nifH1*, *nifD*, and *nifK* genes are transcribed as a single operon. On the other hand, most mutants in the *nifDK* region, and only mutants in this region, fail to show Nif$^+$ complementation when they carry any plasmid subclone. The noncomplementing mutants of the *nifDK* region do not carry additional mutations outside this region because they can be rescued to Nif$^+$ by a double recombinational event involving wild-type plasmid subclones. Indeed, these noncomplementing mutants behave as pseudodominant alleles. Because in all cases studied, nitrogenase component I is tetrameric (D_2K_2) and is the template for assembly of the iron-molybdenum cofactor, the pseudodominance of *nifDK* mutants may be due to interference by mutant peptides with component I assembly or maturation processes.

Nif$^-$ *nifH2*::Vi mutants were not obtained. On the basis of the statistical argument given above, we would have expected to have recovered such mutants. Hypothetically, *nifH2*::Vi mutants might remain Nif$^+$ by virtue of intragenomic *nifH1*$^+$ complementation. If the *nifH2*$^+$ gene proves functional, it must necessarily reside in an operon distinct from that involving the *nif* genes with homology to the *R. meliloti* P2 *fixABC* operon genes. A number of biochemical arguments substantiate the need for a stoichiometric excess of nitrogenase component II, in part because this iron-sulfur protein mediates sequential one-electron transfers to nitrogenase component I. *Azotobacter* and *Rhodopseudomonas* represent two diverse genera of diazotrophs; both *Azotobacter chroococcum* and *Rhodopseudomonas capsulata* contain multiple *nifH* genes (18, 28), as does a fast-growing *R. phaseoli* strain (23).

By any number of biochemical and physiological criteria, rhizobia can be classified as either fast or slow growing and should be considered members of two distinct genera. Although *Rhizobium* sp. strain ORS571 grows fast, it displays physiological properties of the slow-growing group in its carbohydrate metabolism, nodulation properties, lack of detectable plasmids, and ability to fix N_2 ex planta. The organization of the ORS571 *nif* genes, however, is distinct from that of the fast- and slow-growing rhizobia, to the extent that such information is available. In fast-growing rhizobia, such as *R. meliloti* or the *R. leguminosarum-R. phaseoli-R. trifolii* group, a *nifHDK* operon and tightly linked *fixABC-nifAB* genes reside on a symbiotic megaplasmid (2, 4, 10, 19, 23, 31). In slow-growing *R. japonicum* and *Rhizobium* spp. cowpea strains, a genomic *nifDK* operon is separate from that of *nifH* (1, 13, 15, 33). In the major *R. japonicum* Nif locus, *nifB*, and *fixBC* gene homologs have been identified; the *fixA* gene is unlinked (15).

R. meliloti strains conduct N_2 fixation only during symbiosis with *Medicago sativa*. Strikingly, the arrangement of identified *R. meliloti nif* genes resembles a composite of *Rhizobium* sp. strain ORS571 Nif loci 1 and 2. In *R. meliloti* strains, a *nifHDK* operon is separated by approximately 8 kb of DNA from *nifA*, and this interim region houses the P2 *fix* operon (2, 4, 19, 31). Although ORS571 Nif locus 2 and *R. meliloti* share the *nifH* P2 operon organization, the ORS571 *nifH2* gene appears to be oriented in a direction opposite to that of *R. meliloti* strains.

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