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Genetic and Physical Map of the Structural Genes (*nifH,D,K*) Coding for the Nitrogenase Complex of *Rhodopseudomonas capsulata*

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Functional genes coding for the structural components of the nitrogenase complex (*nifH,D,K*) have been cloned on an 11.8-kilobase-pair *Hin*dIII fragment of DNA from the photosynthetic bacterium *Rhodopseudomonas capsulata*. The genes were physically mapped by hybridization of individual cloned *nif* genes from *Klebsiella pneumoniae* and *Anabaena* sp. strain 7120 to Southern blots of *Hin*dIII digests of the cloned *R. capsulata* fragment, after introduction of *Hin*dIII sites into the latter at specified locations by insertion of Tn*5*. Plasmids with the 11.8-kilobase-pair *Hin*dIII fragment containing the Tn*5* insertions were also used for complementation tests with chromosomal Nif$^-$ mutations and for the generation of subfragments to locate those mutations by marker rescue. The *R. capsulata nifH,D,K* genes comprise a single unit of expression, with the same organization and polarity as found in *K. pneumoniae*. However, the *R. capsulata nifH,D,K* fragment did not complement Nif$^-$ point mutations in the corresponding *Klebsiella* genes, and the *Klebsiella nif* genes did not function in *R. capsulata*.

The purple nonsulfur photosynthetic bacterium *Rhodopseudomonas capsulata* has the ability to fix atomospheric dinitrogen (N_2) under anaerobic or microaerobic conditions. The ability of many divergent species of procaryotes to fix nitrogen has been shown to be due to a highly conserved enzyme complex called nitrogenase. This enzyme has been purified from many species. It consists of two main structural components: component I (dinitrogenase), a Mo-Fe protein complex composed of two copies of two different subunits, and component II (dinitrogenase reductase), an Fe protein composed of two copies of a single subunit (15).

The entire gene cluster responsible for nitrogen fixation in *Klebsiella pneumoniae* has been characterized (1, 3). There are a total of 17 *nif* genes on seven or eight operons. The three genes coding for the structural subunits of component I (designated *nifD* and *nifK*) and component II (*nifH*) comprise a single operon transcribed in the direction *H,D,K*. The cloned *K. pneumoniae nifH,D,K* genes have been used as a probe to detect homologous DNA from many nitrogen-fixing species, including *R. capsulata* (18).

In another report (P. A. Scolnik and R. Haselkorn, manuscript in preparation), we describe the use of the *K. pneumoniae nifH,D,K* probe pSA30 (5) to isolate *nif* gene-containing DNA from an *R. capsulata* cosmid library. In the work reported here, we localized the *R. capsulata nifH*, *nifD*, and *nifK* genes by Tn*5* insertion and Southern blot hybridization with individual gene probes. The plasmids with mapped Tn*5* inserts were then mobilized into four Nif$^-$ *R. capsulata* mutants (complementable by the wild-type fragment) and tested for complementation of the Nif$^-$ phenotype. The mutations in these four strains were further mapped by marker rescue, which permitted unambiguous assignment to individual *nif* genes.

MATERIALS AND METHODS

Chemicals and media. RCVB (10) is a minimal medium for *R. capsulata*, RCVBNF is minimal medium lacking ammonia, and T is RCVB in which malate is replaced with 0.2 M Tris (pH 7.5). Minimal medium used for *K. pneumoniae* was NFDM (4) supplemented with 10 μg of L-histidine per ml. Tetracycline hydrochloride was obtained from Pfizer Laboratories, New York, N.Y.; penicillin G and methanesulfonic acid ethyl ester were obtained from Sigma Chemical Co., St. Louis Mo.

Bacterial strains. The wild-type strain of *R. capsulata* is SB1003 (22). *R. capsulata* Nif$^-$ mutants designated J43, J56, J57, J58, J602, J61, and J62 were kindly provided by Judy Wall, University of Missouri, Columbia. Mutants J56, J57, and J602 are linked in recombination tests with the gene transfer agent; the others are unlinked to these three and to each other. We isolated four additional Nif$^-$ mutants (PA1 through PA4) as follows: an overnight culture of SB1003 was washed with T medium and resuspended

in half the original volume. Methanesulfonic acid ethyl ester (15 μl/ml) was added to the suspension, which was incubated for 90 min at 37°C with shaking. The cells were then washed twice with RCVBNF, diluted 20-fold with RCVBNF, and incubated anaerobically in the light at 35°C. Penicillin G (2 U/ml, final concentration) was added when the culture reached logarithmic phase. Small samples (0.1 ml) were plated on RCVB 14 to 20 h after the addition of penicillin. A significant percentage of these colonies were Nif⁻ as judged by replica plating on RCVBNF. *K. pneumoniae* wild-type strain UC139 and Nif⁻ mutant strains UC161 (NifK⁻), UC162 (NifD⁻), and UC164 (NifH⁻) were obtained from Claudine Elmerich, Institut Pasteur, Paris (8).

Plasmids. We used the broad host range cloning vectors pRK290 and pRK292 constructed by Ditta et al. (7). The latter is identical to the former, except that the unique *Bgl*III site has been changed to a *Hin*dIII site. These vectors can be mobilized by the helper plasmid pRK2013, and they confer resistance to 5 μg of tetracycline per ml in *R. capsulata*. Plasmid pRPA5 is pRK292 with an 11.8-kilobase-pair (kbp) *Hin*dIII fragment of *R. capsulata* DNA homologous to the *K. pneumoniae nifH,D,K* probe pSA30 (see below). The 11.8-kbp fragment was subcloned into pRK292 from a cosmid library.

Mapping procedures. Construction of the *R. capsulata* cosmid library will be described elsewhere (P. A. Scolnik et al., manuscript in preparation). The procedure used for Tn*5* mutagenesis of pRPA5 was as described by Berg (2). The presence of two *Hin*dIII sties in Tn*5*, symmetrically located about 1.15 kbp from each end of the 5.7-kbp transposon, facilitated physical mapping and deletion analysis of the *nif* genes. The Tn*5* insertions were mapped by *Hin*dIII digestion of the mutated plasmids followed by agarose gel electrophoresis. Plasmids with a single transposon in the 11.8-kbp insert were identified by retention of the 20-kbp vector fragment and loss of the 11.8-kbp insert fragment. The distances from the Tn*5* insertion site to the ends of the insert were determined by measuring the lengths of fragments b and c (Fig. 2) and then subtracting 1.15 kbp of transposon DNA in each fragment (11). Fragments b and c could be distinguished by Southern hybridization by using as probe a plasmid containing only the right 2.2-kbp *Eco*RI-*Hin*dIII fragment to identify fragment c (data not shown). This procedure was used to map 18 Tn*5* insertions in the 11.8-kbp fragment of pRPA5.

Southern hybridization probes. The mapping of the Tn*5* inserts and the location of individual *nif* genes were done by the method of Southern (21). The specific probe used for *nifH* was pSA30.2, which contains the *Eco*RI-*Bgl*II fragment of pSA30 cloned into pBR322. For *nifD*, the probe was pAn256, which contains a 2.8-kbp *Hin*dIII fragment of *Anabaena* 7120 DNA, which includes all but 100 base pairs from the 5′ end of the *Anabaena nifD* gene (17). The *nifK* probe, pSA30.1, contains an internal *Sal*I fragment from the *nifK* gene of pSA30 cloned into pBR322.

Complementation tests and marker rescue. Plasmids containing the 11.8-kbp fragment, its derivatives containing Tn*5*, or its subfragments cut from the Tn*5*-containing derivatives, were maintained in *Escherichia coli* HB101. The mobilizing plasmid pRK2013 was also maintained in *E. coli* HB101. For mobilization, two strains carrying, respectively, pRK2013 and pRPA5 or one of its derivatives were grown overnight in Luria broth medium (14), and 0.02 ml of each was mixed and added to 0.10 ml of the *R. capsulata* Nif⁻ recipient grown overnight in RCVB. Samples (0.02 ml) of the mixture of cells were spotted on RCVB plates, allowed to dry, incubated for 6 h at 37°C and then respread with 0.2 ml of RCVB. Finally, the plate was overlaid with RCVB soft agar containing enough tetracycline to give a final concentration in the plate of 5 μg/ml. After several days, at least three independent tetracycline-resistant colonies were resuspended in 1.0 ml of RCVB and then diluted and plated on RCVBNF and RCVB tetracycline. RCVBNF plates were used to test the Nif phenotype, and RCVB tetracycline plates were used to determine the total number of viable cells containing plasmids. For a positive complementation test, all of the colonies were large and dark red-brown on RCVBNF after 4 days of anaerobic incubation in the light at 35°C. Marker rescue usually produced Nif⁺ colonies at a frequency of 0.5 to 1% of the cells originally Tet^r, whereas reversion was less than 1 in 10^5.

RESULTS

To identify *nif* genes in *R. capsulata*, we used the probe pSA30 (5) which contains the *Klebsiella nifH,D,K* genes. Hybridization of ^{32}P-labeled pSA30 DNA to blots of total *R. capsulata* DNA digested with *Hin*dIII showed two fragments of lengths 11.8 and 4.8 kbp that contain homologous sequences. The isolation of these fragments from recombinant lambda and cosmid libraries will be described elsewhere (P. A. Scolnik et al., manuscript in preparation). The two *Hin*dIII fragments were subcloned from cosmids into pRK292 and mobilized into seven Nif⁻ mutants obtained from Judy Wall, representing five different gene transfer agent linkage groups, and into four Nif⁻ mutants (PA1 through PA4) isolated as described in Materials and Methods. None of the mutants was complemented by the 4.8-kbp *Hin*dIII fragment. It will be shown elsewhere (P. A. Scolnik et al., manuscript in preparation) that this fragment contains *nif* gene-related sequences that are not normally expressed in *R. capsulata*. Mutants J56, J57, J602, and PA1 were complemented by the 11.8-kbp *Hin*dIII fragment. Mutants J56, J57, and J602 are members of gene transfer agent group IV, which will be shown below to consist of genes *nifH*, *nifD*, and *nifK*. Mutants J43, J58, J61, J62 (gene transfer agent groups I, II, III, and V), and PA2 through PA4 were not complemented by the 11.8-kbp fragment.

Localization of *nifH,D,K*. A total of 18 plasmids with Tn*5* insertions in the 11.8-kbp *Hin*dIII fragment in pRPA5 were isolated and physically mapped as described in Materials and Methods (Fig. 1). Southern blot analysis was then used to locate the regions of homology between the 11.8-kbp fragment and individual *nif* genes from other species (Fig. 2). Insertions 5.05 through

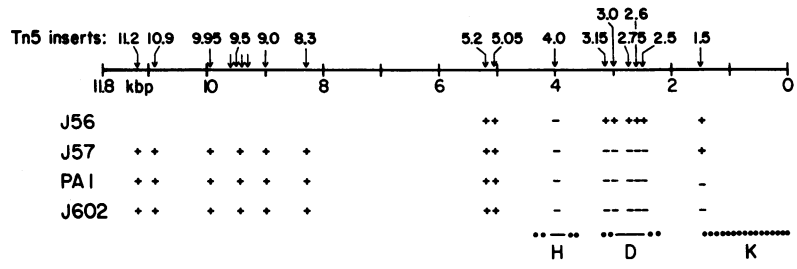

FIG. 1. Tn*5* insertion mapping and mutagenesis of the *nif* region of *R. capsulata*. A total of 18 independent Tn*5* inserts were mapped within the 11.8-kbp *Hin*dIII fragment as described in the text and named according to their distance in kbp from the right-end *Hin*dIII site, as shown. The results of the complementation tests with each of the four chromosomal Nif$^-$ mutants in the left column are shown for each insert, with a + indicating complementation. Horizontal lines below the complementation results indicate the regions of certain homology with *nif* gene-specific probes, as determined in Fig. 2. Black dots indicate regions of homology whose precise boundaries are unknown. At 9.5, there are four closely spaced insertions, each of which gave the same complementation result.

1.5 were cut with *Hin*dIII, fractionated by agarose gel electrophoresis, and blotted onto nitrocellulose (Fig. 2). Three blots of the cut inserts were hybridized to ^{32}P-labeled DNA containing *nifH*, *nifD*, or *nifK* probes (Fig. 2). Ignoring the homology between vectors (top band), *nifH* hybridized to the right of insert 5.05, on either side of insert 4.0, and to the left of insert 3.15. *Anabaena nifD* hybridized to the right of insert 3.15 and to the left of insert 1.5. Finally, the *nifK* probe hybridized only to the right of insert 1.5. Hybridization data of this type cannot determine the precise boundaries of the genes because the ends of the genes may not be homologous between species. However, we can conclude that the *R. capsulata nifH*, *nifD*, and *nifK* genes have

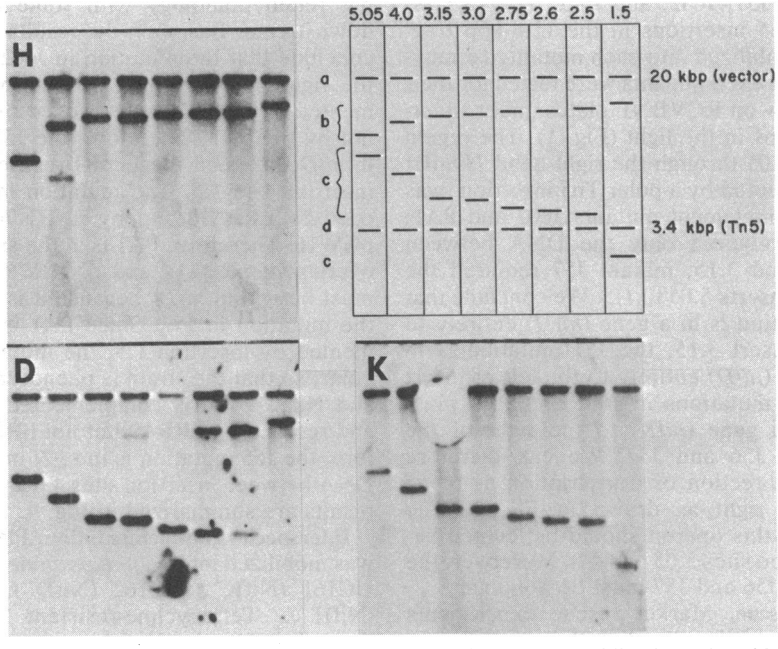

FIG. 2. Physical mapping of the *nif* genes of *R. capsulata* by Southern hybridization. Plasmids containing the Tn*5* inserts to the right of 5.2 in Fig. 1 were cut with *Hin*dIII and fractionated by electrophoresis on three agarose gels. A diagram of the staining pattern is shown in the upper right panel. The 20-kbp fragment (a) is the vector; the 3.4-kbp fragment (d) is the internal part of Tn*5*. Fragment (b) is from the left of the insert and fragment (c) is from the right. Three identical gels were blotted and probed individually with *nif* gene-specific plasmid DNA as described in the text. The vector band lights up due to homology with the plasmid probes. Low intensity of band b in the *nifD*-probed insert 2.75 is probably due to poor DNA transfer in that region.

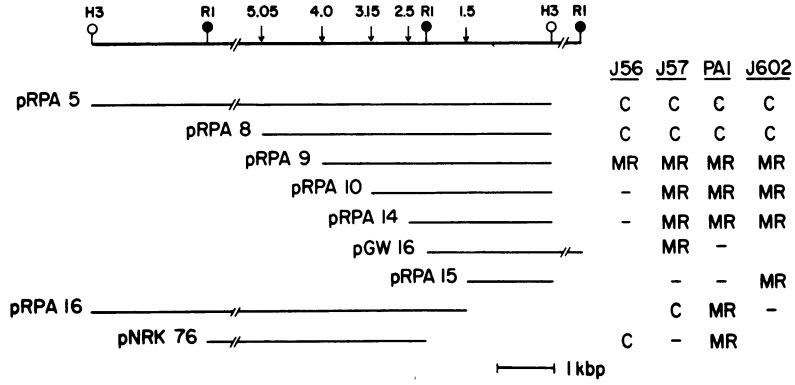

FIG. 3. Deletion mapping of Nif⁻ mutations in *R. capsulata*. The pRPA plasmid series was prepared by subcloning *Hin*dIII fragments from the plasmids with Tn*5* inserts shown on the map in Fig. 1. Plasmids pGW16 and pNRK76 contain *Eco*RI fragments isolated from a lambda library. The table on the right indicates the results of crosses between the plasmids containing wild-type DNA fragments and the mutants shown. Symbols: C, complementation; MR, marker rescue; —, neither complementation nor marker rescue. No entry means the experiment was not done.

the same order as in *K. pneumoniae* and that they are located within a DNA fragment no greater than 5.05 kbp.

Tn*5* mutagenesis. The Tn*5* insertions were then used to identify the region of pRPA5 required for complementation of the four Nif⁻ mutants J56, J57, J602, and PA1. The 18 plasmids with Tn*5* insertions in the 11.8-kbp fragment were mobilized into each mutant. Tetracycline-resistant exconjugants were tested for their ability to grow on RCVBNF plates under anaerobic conditions in the light (Fig. 1). The region from insert 5.05 through the right-hand *Hin*dIII site, uninterrupted by a polar Tn*5* insertion, was needed to complement mutants J602 and PA1; mutant J56 required only the DNA between insert 5.05 and 3.15; mutant J57 required the region from inserts 5.05 to 1.5. We conclude that the J56 mutation is in a gene (*nifH*) entirely to the left of insert 3.15, the J57 mutation is in another gene (*nifD*) entirely to the left of insert 1.5, and the mutations in J602 and PA1 must affect a third gene (*nifK*) to the right of the mutations in J56 and J57. We can therefore identify the direction of transcription as being from left to right as drawn in Fig. 1. The promoter for this operon should be located between insertion sites 5.05 and 4.0. Moreover, the mutations in J56 and J57 must be nonpolar.

Marker rescue. Marker rescue experiments were used to further localize the mutations in J56, J57, J602, and PA1. Marker rescue is easily distinguished from complementation because marker rescue depends on recombination which occurs at a very low frequency, whereas complementation leads to 100% of the tetracycline-resistant recipients being Nif⁺. The *Hin*dIII sites introduced into pRPA5 by the insertion of Tn*5* were used to subclone portions of the 11.8-kbp fragment into pRK292 (Fig. 3). Plasmid pRPA8 contains the entire *nifH,D,K* operon and is able to complement all four mutants. Plasmids pRPA9 through pRPA15 all lack the promotor but retain homology with some of the genes downstream. Based on the results in Fig. 3, we conclude that the mutation in J602 is in *nifK* to the right of insert 1.5. Mutant J57 is complemented by pRPA16 and rescued by pGW16 but not by pRPA15. Therefore, the J57 mutation is in *nifD*, between the *Eco*RI restriction site and insertion site 1.5. The mutation in PA1 is rescued by pRPA14 and by pNRK76 but not by pGW16. Therefore, PA1 is in the small region of overlap of pRPA14 and pNRK76. This region must be within *nifD*, because it is to the left of the mutation in J57. Since PA1 is not complemented by insertion 1.5, the mutation must be polar, so that the strain is phenotypically NifD⁻ and NifK⁻. J56 is complemented by pNRK76 and rescued by pRPA9 but not pRPA10. Therefore, the J56 mutation is in *nifH* in the region of DNA between insertion sites 4.0 and 3.15. These results are summarized in Fig. 4.

Interspecies complementation. Plasmid pRPA8 was mobilized into *K. pneumoniae* Nif⁻ mutants UC161 (NifK⁻, UC162 (NifD⁻), and UC164 (NifH⁻). Tetracycline-resistant exconjugants were found to be unable to grow on NFDM under anaerobic conditions. The *K. pneumoniae nifH,D,K* operon from pSA30 was subcloned into pRK290 and crossed into *R. capsulata* mutants J57, J602, and PA1 as well as *K. pneumoniae* mutant strains UC161, UC162, and UC164. The *nifH,D,K* operon from *K. pneumo-*

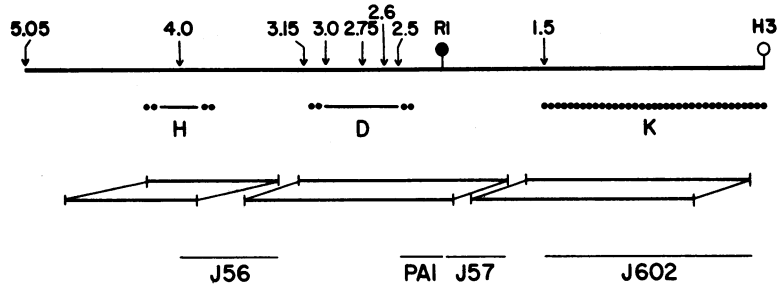

FIG. 4. Summary of mapping information for the *nif* genes of *R. capsulata*. The upper line shows the rightmost 5.05 kbp of the 11.8-kbp *Hin*dIII fragment (Fig. 1). The lines below indicate regions of homology with heterologous probes (Fig. 1 and 2). The lines below them indicate the extreme left and right boundaries for the three *nif* genes determined by hybridization, complementation, and marker rescue. The limits defined by marker rescue for the four Nif⁻ mutations are also shown. The lengths of the genes are based on the molecular weights of the gene products (9), which predict 900 base pairs for *nifH*, 1.4 kbp for *nifD*, and 1.5 kbp for *nifK*. It is assumed that there are 115 base pairs between genes. The only intergene spaces actually determined are 115 base pairs between *nifH* and *nifD* in *Anabaena* sp. strain 7120 (13) and 13 base pairs between *nifH* and *nifD* in *K. pneumoniae* (20).

niae was able to complement the Nif⁻ phenotype of each of the *Klebsiella* mutants but none of the *R. capsulata* mutants.

DISCUSSION

Conservation of nucleotide sequence has made possible the cloning and physical mapping of the *nifH*, *nifD*, and *nifK* genes from a wide variety of bacteria. Thus far, two different organizations of these genes have been described. The archetype is that of *K. pneumoniae*, in which the three genes are transcribed together from a single promoter next to *nifH* (1, 3). A similar organization appears to be found in *Rhizobium meliloti* (6, 19), *Azospirillum brasilense* (16), and the unicellular cyanobacterium *Gloeothece* (12). In the filamentous cyanobacterium *Anabaena* sp. strain 7120, *nifH* and *nifD* are adjacent and cotranscribed, whereas *nifK* is 11 kbp from *nifD* and transcribed independently (17).

The results reported here indicate that the organization of *nifH,D,K* in *R. capsulata* is similar to that in *K. pneumoniae*. The hybridization results, using Tn*5* to split individual genes, show that the order is *nifH*, *nifD*, and *nifK*. Since insertion of Tn*5* at 4.0, within *nifH*, destroys the ability to complement mutant J602, which is in *nifK*, the direction of transcription must be *nifH*→*nifK*, as in *K. pneumoniae*.

Complementation experiments with the Tn*5*-mutated 11.8-kbp *Hin*dIII fragment mobilized into the Nif⁻ mutants allowed us to identify three functional *nif* genes on a single operon. Insertion 4.0 inactivates a gene required to complement mutant J56, insertions 3.15 through 2.5 inactivate a region required to complement J57, and insertion 1.5 inactivates a third gene required to complement J602 and PA1. Marker rescue with fragments of DNA showed that J602 is in *nifK*. This genetic evidence is in agreement with the physical evidence from hybridization studies and allows us to conclude that the mutation in J56 is in *nifH*, the mutation in J57 is in *nifD*, and the mutation in J602 is in *nifK*. We can also conclude that the mutations in J56 and J57 are nonpolar because in both cases the region downstream in the complementing plasmid can be mutated by Tn*5* insertion and still retain the ability to complement. The mutation in PA1 maps between insertion 2.5 and the *Eco*RI site at 2.2, i.e., within 300 base pairs in the *nifD* gene. It must be a polar mutation to explain the failure to be complemented by insertion 1.5, which is in the *nifK* gene.

Based on the hybridization results and the known molecular weights of the *nifH*, *nifD*, and *nifK* gene products (9), it is possible to locate the genes with some accuracy on the physical map (Fig. 4). The 3′ end of the *nifD* gene must be to the right of the *Eco*RI site at 2.2 because the *Eco*RI 7.6-kbp fragment does not complement or rescue mutant J57, whereas the adjacent *Eco*RI fragment (pGW16) rescues the mutant. The 5′ end of *nifK* must be to the left of 1.5 because the coding region of the gene requires more than 1.5 kbp. Therefore, the 5′ end of *nifK* must map between 1.6 and 2.0 kbp from the *Hin*dIII site, and the 3′ end of *nifD* must map between 1.7 and 2.1 kbp from the *Hin*dIII site. Given the size of the *nifK* gene, its 3′ end must be within 500 base pairs of the *Hin*dIII site, which does not leave room for another gene downstream of *nifK* on the 11.8-kbp *Hin*dIII fragment. To the left, the 5′ end of *nifD* must map between 3.15 and 3.55 kbp from the *Hin*dIII site. For *nifH*, the only other restriction is that the Tn*5* insert 4.0 be within the gene. Therefore, the 3′ end could be anywhere

between 3.3 and 3.9 kbp and the 5′ end between 4.2 and 4.8 kbp from the *Hin*dIII site.

An unresolved question is the failure of interspecific complementation between *R. capsulata* and *K. pneumoniae*. The 11.8-kbp fragment from *R. capsulata* failed to complement mutants of *K. pneumoniae* that were complemented by the 6.2-kbp *Eco*RI *Klebsiella* fragment from pSA30 recloned into pRK290. The latter plasmid failed to complement J57, PA1, and J602. Since both plasmids contain promoter and genes coding for the entire nitrogenase complex, failure to complement cannot be due to poor component interactions but must reflect either transcription failure or poor "maturation" of the enzyme components.

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