Mol Gen Genet (1985) 201:363–369

MGG

© Springer-Verlag 1985

Isolation and organization of genes for nitrogen fixation in *Rhodopseudomonas capsulata*

Peter Avtges, Robert G. Kranz, and Robert Haselkorn
Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637, USA

Summary. A library of *Rhodopseudomonas capsulata* chromosomal DNA was constructed in the broad host range cosmid vector pLAFR1. The library was used to isolate nitrogen fixation genes by complementation of *R. capsulata* Nif$^-$ mutants. Four complementing regions were localized on different cloned DNA fragments by Tn*5* and mini-Mu mutagenesis. Additional *nif* genes were identified by recombination of transposons from the *nif* cosmids into the *R. capsulata* chromosome resulting in the creation of new Nif$^-$ mutations. Most of the newly cloned DNA fragments containing *nif* genes were found to be unlinked to any other by Southern hybridization of the cloned DNA to chromosomal DNA blots. One of the new fragments was linked to the *nifHDK* genes. Another cluster spanning 10–12 kilobase pairs contained a number of *nif* genes, possibly as many as eight.

Introduction

Nitrogen fixation in microorganisms is a complex process requiring some 15–17 genes. In *Klebsiella pneumoniae* these genes are clustered within 24 kilobase pairs on the chromosome and transcribed in seven or eight operons (Ausubel and Cannon 1980; Brill 1980). A suggestion that the organization of *nif* genes in *R. capsulata* differs from that of *K. pneumoniae* came from the results of gene transfer agent (GTA) mediated recombination among independently isolated Nif$^-$ mutations, which were found to comprise five (Wall and Braddock 1984) and later six (Wall et al. 1984) GTA linkage groups. Since the GTA packages 4.6-kb fragments of linear DNA, two mutations separated by 4.6 kb appear unlinked. In practice, GTA linkage markers can be established reliably only if they are within 2.7 kb. Wall and Braddock (1984) point out that their GTA mapping of the *R. capsulata nif* genes is not inconsistent with a single cluster covering 30 kb. In addition, while our work was in progress, Pühler et al. (1984) identified a 14 kb region of the *R. capsulata* chromosome that appeared to be saturated with *nif* genes. It was therefore of interest to map physically the *nif* genes in the various GTA linkage groups (Wall and Braddock 1984), to compare the mapped DNA fragments with those of Pühler et al. (1984), and finally to compare the *nif* gene organization of *R. capsulata* with that of *Klebsiella* (Ausubel and Cannon 1980; Brill 1980).

Offprint requests to: R. Haselkorn

We have previously shown that an 11.8-kb *Hin*dIII fragment of wild type *R. capsulata* DNA, cloned on the basis of homology with *Klebsiella* genes coding for the nitrogenase components (*nifHDK*), complements *R. capsulata* Nif$^-$ mutants in a single GTA group (IV). In this paper we report the isolation of DNA fragments containing a large number of *R. capsulata nif* genes. Tn*5* and mini-Mu insertional inactivation of complementing activities, chromosomal mutagenesis with the Tn*5*-containing fragments, and Southern blots of chromosomal DNA probed with the complementing fragments were carried out. These results show that in *R. capsulata*, *nif* genes are widely dispersed among non-*nif* genes.

Materials and methods

Chemicals and media. RCVB (Johansson and Gest 1977) is a minimal medium for *R. capsulata*; RCVBNF is minimal medium lacking ammonia. Antibiotics of the following concentrations were added to RCVB plates: 2.5 μg/ml tetracycline (Tc), 15 μg/ml kanamycin (Km), 5 μg/ml gentamicin (Gm). LB medium was used for *E. coli* (Miller 1972).

Bacterial strains. The wild type strain of *R. capsulata* was SB1003 (Yen and Marrs 1976). Nif$^-$ mutants designated J43 (GTA I), J61 (GTA III), J62 (GTA II) and LJI (GTA VI) were kindly provided by Judy Wall, University of Missouri, Columbia, MO, USA. Nif$^-$ mutants AH1 and AH3 were kindly provided by Ayala Hochman, Tel Aviv University, Israel. The Nif$^-$ mutant PA3 was isolated by us as previously described (Avtges et al. 1983). *E. coli* strain HB101 was also previously described (Boyer and Rouland-Dussoix 1969).

Plasmids. We used the broad host range cosmid pLAFR1 (21.6 kb) which carries a Tc^r marker (Friedman et al. 1982). This cosmid was constructed from plasmid pRK290 (Ditta et al. 1980) by the addition of a *cos* site. It is mobilized from *E. coli* HB101 into *R. capsulata* by three factor crosses with the helper plasmid pRK2013 (Ditta et al. 1980), which carries Km^r. Plasmid pPH1 is incompatible with pLAFR1 and carries a gentamicin resistance gene (Hirsch 1984). Plasmid pRPA5 (Avtges et al. 1983) carries the *nifHDK* genes of *R. capsulata* in pRK292, a derivative of pRK290.

Construction of cosmid library. Chromosomal DNA was partially digested with *Eco*RI to an average size of

20–50 kb, then ligated to pLAFR1 digested to completion with *Eco*RI, and packaged in lambda particles in vitro as described (Enquist and Sternberg 1979). Packaged particles were used to infect *E. coli* HB101, which were plated on LB+Tc (20 µg/ml). The resulting library consisted of approximately 5,000 colonies. Sampling these colonies indicated that 5 of 11 had 0 to 10 kb inserts, 3 of 11 had inserts of 10 to 20 kb, and 3 of 11 had inserts of 30 kb. Cosmids with inserts less than 25 kb contain two copies of the vector.

Isolation of nif cosmids. Cosmids with *nif* genes were isolated as follows: 5 volumes of an overnight culture of a Nif^- *R. capsulata* mutant was mixed with 1 volume each of overnight cultures of HB101 (cosmid library) and HB101 (pRK2013) grown on LB without drug. The cells were concentrated by centrifugation and then spotted on an RCVB plate, which was incubated in the dark overnight. The cells were then resuspended in RCVB, respread on RCVB-Tc plates, and incubated 4–5 days aerobically in the dark after which about 5,000 colonies/plate were visible. The efficiency of plasmid transfer was variable; in most experiments it was about 10^{-6} per recipient. The Tc^r *R. capsulata* exconjugants were replica-plated onto RCVB-NF plates and incubated anaerobically in the light until Nif^+ colonies were visible. The frequency of Nif^+ colonies ranged between 0.1 and 1%. Plasmid DNA was prepared immediately from these colonies and used to transform *E. coli* HB101. The presence of Nif complementing activity in the Tc^r transformants was confirmed by conjugation back into the original Nif^- mutant, yielding 100% Nif^+ colonies. Complementation is readily distinguished from marker rescue because the latter occurs less than 2% as frequently as complementation (Avtges et al. 1983; Scolnik et al. 1983).

Physical mapping of complementing nif genes by Tn5 and mini-Mu insertion. Transposon Tn*5* was inserted in each of the cosmids isolated by complementation, as described by Ruvkun and Ausubel (1981). Mini-Mu *d*II1734 inserts were obtained as described by Castilho et al. (1984). Each insert was mapped physically by digesting plasmid DNA preparations with restriction enzymes and hybridization with specific probes when necessary. The ability of each mini-Mu or Tn*5*-containing cosmid to complement the original Nif^- mutant was then determined by conjugation from HB101 back into the *R. capsulata* mutant, as described above.

Tn5 mutagenesis of the R. capsulata chromosome. This procedure followed the design of Ruvkun and Ausubel (1981). Each of the cosmids containing Tn*5* inserts was mobilized into wild type *R. capsulata* SB1003, using pRK2013 as described above. The exconjugants were selected on RCVB-Tc plates and then transferred onto RCVB-Km plates. Colonies appearing on the latter plates should contain only the Tn*5* mutagenized cosmid, since the cosmid vector carries Tc^r and the Tn*5* carries Km^r. Cells from individual colonies were then crossed with *E. coli* HB101 carrying the plasmid pPH1, which is incompatible with pRK290 derivatives and contains a Gm^r gene. Exconjugants from this cross were selected on RCVB containing both Km and Gm. Km^r Gm^r colonies from the selection plate were transferred to RCVB-Tc (to verify that the cosmid was gone), RCVB-Km (to verify that Tn*5* was still present), and RCVB-NF (to determine the Nif phenotype). Nif^+ colonies were re-tested on RCVB-Tc and RCVB-Km. Chromosomal DNA was prepared from all strains in which Tn*5* appeared to be transferred to the chromosome, digested with *Eco*RI, and analyzed by Southern hybridization using the *Eco*RI fragment from the cosmid that had originally been mutagenized as a probe. The Nif phenotypes are reported only for strains in which the blot showed Tn*5* uniquely inserted in the correct *Eco*RI fragment, the cosmid bands absent, and pPH1 present.

Results

We isolated, by complementation of individual Nif^- mutations, wild type *R. capsulata* DNA fragments containing the corresonding *nif* genes. This aproach allowed us to identify fragments complementing mutations J43 (GTA group I), J61 (GTA group III), PA3 (GTA group unknown) and LJ1 (and AH1 and AH3) (Avtges et al. 1983; Wall and Braddock 1984). In addition, we were able to locate mutation J62 (GTA group II) by marker rescue.

For each Nif^- mutation, a cosmid with a fragment of wild-type *R. capsulata* DNA was identified by complementation with a cosmid library. The region of each fragment needed for complementation was then localized by insertion of transposon Tn*5* or mini-Mu and observation of the Nif phenotype when the transposon-containing fragment was reintroduced into the original mutant by plasmid conjugation. Next, a number of Tn*5*-containing fragments were introduced into wild-type *R. capsulata* on plasmids and forced to recombine with the chromosome. Some of these chromosomal insertions resulted in the creation of new Nif^- mutations. Finally, the DNA fragments found in the original complementing cosmids were used as probes in Southern blots of total *R. capsulata* DNA digested with several restriction enzymes, in order to determine physical linkage, if possible, among the complementing DNA fragments. Figure 1 shows maps of the clones which were isolated and the results of the Tn*5* and mini-Mu complementation analysis. Also shown are phenotypes of the Tn*5* chromosomal insertions. The location of each Tn*5* chromosomal recombinant was verified by Southern hybridization using the original complementing *Eco*RI fragment as the probe. These blots are shown in Fig. 2.

Mutant J43

The *R. capsulata* Nif^- mutant J32 was complemented by a cosmid designated pRCN101, which contains two *Eco*RI fragments of length 3.55 and 3.75 kb. Figure 1 A shows the results of J43 complementation tests using various Tn*5* insertions in pRCN101. The complementing activity requires no more than the region between 0.5 and 2.0 kb from the left end of the 3.75 kb fragment, or 1.5 kb. Since Tn*5* insertions are polar, this result suggests that the gene mutated in J43 is transcribed either by itself, in either direction, or as the promoter-proximal gene of a small operon, from left to right. When recombined into the chromosome, Tn*5* inserts at 2.0 and 2.7 kb resulted in a Nif^- phenotype. However, since inserts at these positions did not affect the J43 complementing activity of pRCN101, the gene(s) inactivated by the inserts cannot lie between J43 and its promoter.

All of the Tn*5* inserts in the 3.55 kb fragment of pRCN101 (the right most fragment in Fig. 1 A), recombined

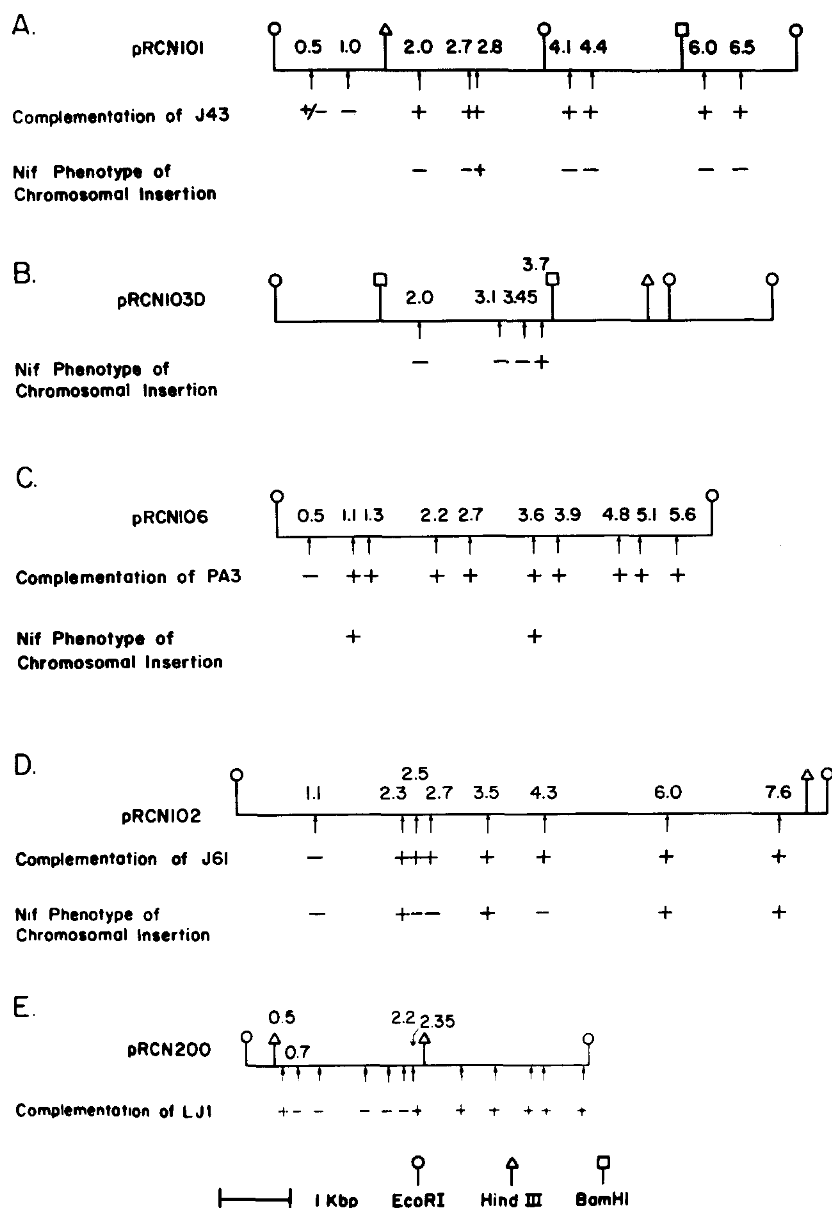

Fig. 1A–E. Physical maps and Tn*5* or mini-Mu insert locations for *R. capsulata* DNA fragments containing *nif* genes. For each fragment except the ones cloned in pRCN103D, the ability of each transposon-inserted fragment to complement the Nif$^-$ mutation is indicated by a + or −. Wherever a Tn*5* was recombined into the chromosome, the Nif phenotype of the resulting strain is given by a + or −. Absence of a symbol means that chromosomal inserts were not recovered at that location. All of the inserts are Tn*5*, except those in pRCN 200, which are mini-Mu. Numbers over each *vertical arrow* correspond to the distance between the site of that insert and the left-hand *Eco*RI site. The +/− for insert 0.5 in pRCN101 means very weak complementation, i.e. poor growth on RCVBNF

into the chromosome, result in a Nif$^-$ phenotype. The minimum size of the *nif* gene region defined by these inserts is 2.4 kb, but it could be much larger, extending as far as the insert at 2.8 on the left, and off the cloned fragment to the right.

Mutant J62

Attempts to isolate a cosmid complementing the Nif$^-$ mutant J62 were not successful. Nif$^+$ colonies of *R. capsulata* were isolated from the original conjugation with the cosmid library, but when the resulting cosmid was used to transform *E. coli* HB101 and then re-tested in J62, only Nif$^-$ colonies were found. This procedure resulted reproducibly in the isolation of a cosmid designated pRCN103D, which contained two *Eco*RI fragments of 5.5 and 1.45 kb (Fig. 1B). This suggests that one of these fragments contains part of the gene mutated in J62 and that the original Nif$^+$ colonies result from a reciprocal exchange between the cosmid and the chromosome. Such exchanges would make the chromosome wild-type but the cosmid would then contain the original J62 mutation and consequently be unable to "complement" J62 when re-tested. It was, therefore, not possible to locate the region of pRCN103D that includes the J62 mutation using Tn*5* insertions. However, Tn*5* inserts in the 5.5-kb *Eco*RI fragment of pRCN103D, when recombined into the chromosome, result in Nif$^-$ mutations (Fig. 1B). *Nif* genes are found in a region of at least 1.45 kb in the middle of the fragment. pRCN103D complements the Nif$^-$ mutations created by recombination of Tn*5* inserts 2.0, 3.1 and 3.45 into the chromosome, indicating that the promoters for the genes so mutated lie within the fragments cloned in pRCN103D. We will show below that pRCN101 and pRCN103D hybridize to identical *Hin*dIII and *Bam*HI fragments in total *R. capsulata* DNA and therefore the *Eco*RI fragments in the two cosmids are neighbors in the chromosome.

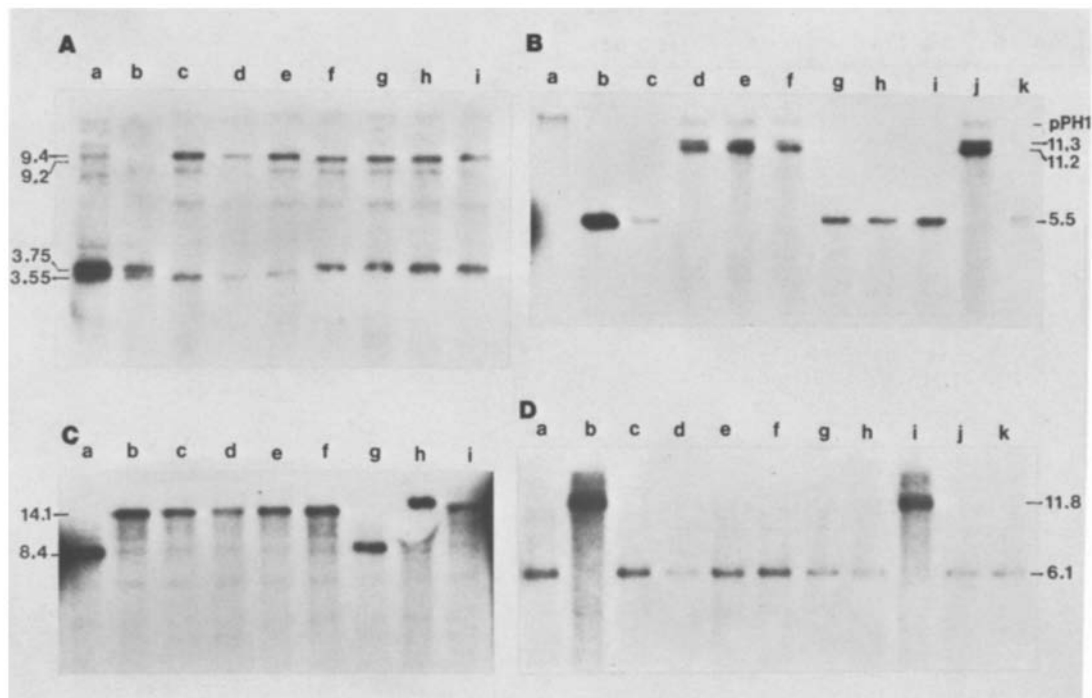

Fig. 2A–D. Southern hybridization confirming the recombination of Tn*5* into the chromosome of wild type *R. capsulata* at defined locations. Each panel shows total DNA from *R. capsulata* digested with *Eco*RI and probed with the *Eco*RI fragments from the original complementing cosmid labeled by nick-translation. **A** pRCN101, **B** pRCN103D, **C** pRCN102, **D** pRCN106. In each panel, one lane contained wild type *R. capsulata* DNA: **A**, lane a; **B**, lane b; **C**, lane a and **D**, lane a. Panel **B**, lane a contained pPH1 DNA. Panel **A**, lane b shows the failure to recombine insert 1.0 of pRCN101 into the chromosome. Lanes c, d, and e show Tn*5* inserted into the 3.75 kb *Eco*RI fragment (inserts 2.0, 2.7, 2.8); lanes f, g, h, and i show Tn*5* inserted into the 3.55 kb fragment (inserts 4.1, 4.4, 6.0 and 6.5 of Figure 1A). Panel **B** shows the chromosomal insertion of Tn*5* at positions 2.0, 3.1, 3.45 and 3.7 in lanes d, e, and j, respectively. The other lanes show five other Tn*5* inserts that failed to enter the chromosome from pRCN103D. The doublets at 11.3 and 11.2 kb are due to partial digestion of the chromosomal DNA; note that pRCN103D contains a 1.45 kb *Eco*RI fragment adjacent to the 5.5 kb one. Panel **C** shows that all of the Tn*5* inserts in pRCN102 were recombined into the chromosome except 4.3 (lane g). This chromosomal insert was obtained subsequently (data not shown). Panel **D** shows the results for pRCN106, in which only inserts 1.1 and 3.6 (lanes b and i) were recovered as chromosomal insertions. The other eight lanes correspond to the eight other Tn*5* inserts in Fig. 1C. Since the recovery of chromosomal inserts was so infrequent for pRCN106 compared to the other cosmids, we suspect that pRCN106 contains one or more genes essential for growth on RCVB

Mutant J61

The mutation J61 was found by Wall and Braddock (1984) to be unlinked to other Nif$^-$ mutations that were tested and to result in the absence of the nitrogenase structural proteins. An 8.4 kb *Eco*RI fragment of wild-type *R. capsulata* DNA containing a gene complementing the J61 mutation was isolated in cosmid pRCN102 (Fig. 1D). Elimination of the complementing activity by Tn*5* insertion shows the gene to be somewhere within the left 2.3 kb of the fragment. Recombinational insertion of Tn*5* from positions 2.5 and 2.7 of the cosmid into the chromosome indicate that a second *nif* region is located on the *Eco*RI fragment. A third *nif* gene(s) lies between 3.5 and 6.0 since the Tn*5* at position 4.3 also results in a Nif$^-$ phenotype. Tn*5* insertions between these three regions (at 2.3 and 3.5) result in Nif$^+$ phenotypes.

Mutant PA3

Mutant PA3 was complemented by cosmid pRCN106, which contains a 6.1-kb *Eco*RI fragment of *R. capsulata* DNA. Only one of the ten Tn*5* inserts in this fragment eliminated the complementing activity (Fig. 1C), indicating that the gene responsible is no greater than 1 kb. When two of the Tn*5* inserts were transferred to the chromosome by recombination, no Nif$^-$ mutants were recovered. With the other Tn*5* inserts in pRCN106, a few colonies of Km^r Gm^r Tc^s cells are obtained, but these contained neither Tn*5* nor pH1 (see Fig. 2D) and therefore must have been spontaneous Km^r Gm^r mutants.

Mutant LJ1 (and AH1 and AH3)

The final set of complementations was done with the Nif$^-$ mutants LJ1, AH1 and AH3. For all three strains, each Nif$^+$ exconjugant yielded the same cosmid, pRCN200, containing a 4.8 kb *R. capsulata Eco*RI fragment. In order to map the gene(s) on this fragment, Mu*d*II1734 inserts were constructed. Complementation analysis of 22 mini-Mu inserts indicate a minimum size of 1.5 kb and maximum size of 1.9 kb for the LJ1 gene. Only 12 of these inserts are shown in Fig. 1E. Complementation of AH3 was inactivated by the same inserts that inactivated LJ1 complementation, suggesting that this mutation is in the same gene as LJ1.

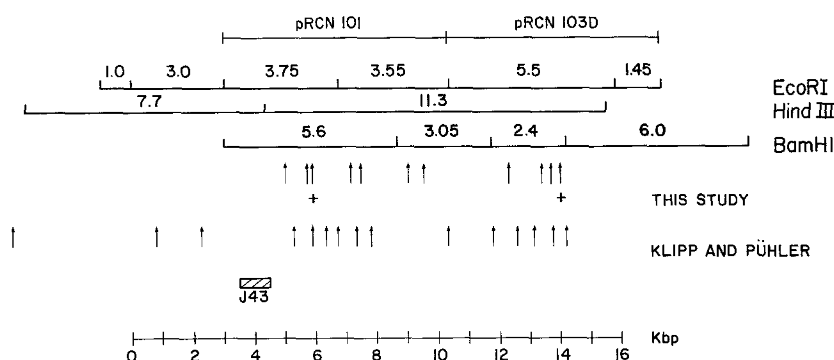

Fig. 3. Physical map and Tn*5* insert locations for the *R. capsulata nif* gene region surrounding mutations J43 and J62. Restriction sites were located on the cloned fragments and the maps aligned as described in the text. *Arrows* indicate the approximate locations of Tn*5* inserts determined by us (Fig. 1) and by Pühler et al. (1984). All of the chromosomal Tn*5* inserts result in a Nif⁻ phenotype except for the two marked with a (+) sign. The shaded bar shows the approximate location of the mutation in J43

Table 1. Hybridization analysis of restriction fragments in cosmids that complement *R. capsulata* Nif⁻ mutants

Mutation	Cosmid	Complementing fragment[a]	Related fragments[b]
J56 (*nifH*)	pRPA5	*Hin*dIII 11.8	*Eco*RI 7.6, 5.0, 3.7 *Bam*HI 20
PA1, J57 (*nifD*)	pRPA5	*Hin*dIII 11.8	*Eco*RI 7.6, 5.0, 3.7 *Bam*HI 20
J60 (*nifK*)	pRPA5	*Hin*dIII 11.8	*Eco*RI 7.6, 5.0, 3.7 *Bam*HI 20
J61	pRCN102	*Eco*RI 8.4	*Hin*dIII 8.2 *Bam*HI 7.0, 2.0
J43	pRCN101	*Eco*RI 3.75	*Eco* RI 3.55 *Hin*dIII 11.3, 7.7 *Bam*HI 5.6, 3.05
J62	pRCN103D	–	*Eco*RI 5.5, 1.45 *Hin*dIII 11.3 *Bam*HI 6.0, 3.05, 2.4
PA3	pRCN106	*Eco*RI 6.1	*Eco*RI 9.0, 6.5, 5.6[c] *Hin*dIII 19 *Bam*HI 15
LJ1, AH1, AH3	pRCN200	*Eco*RI 4.8	*Hin*dIII 13.0, 5.8, 2.1 *Bam*HI 20

[a] Size, in kb, of the restriction fragment within which the region complementing the mutation in the first column is located

[b] Size, in kb, of other fragments in the complementing cosmid or of chromosomal DNA fragments that hybridize with the complementing cosmid fragments. Underlining indicates the fragments of pRPA5 that contain the H, D and K genes, respectively (Avtges et al. 1983)

[c] These *Eco*RI fragments were present on another cosmid which also contained the 6.1-kb *Eco*RI fragment complementing mutant PA3

Southern hybridization with the cosmid probes

Each of the *Eco*RI inserts in the cosmids pRCN101, pRCN103D, pRCN102, pRCN106 and pRCN200 was labeled by nick-translation and used as probe in Southern hybridization with total *R. capsulata* DNA digested with *Eco*RI, *Hin*dIII, or *Bam*HI (Table 1). Each *Eco*RI digest gave the expected results, i.e. only the bands corresponding to the probes were identified, indicating that none of the genes isolated is present in extra or multiple copies. Table 1 also includes the data for similar hybridizations done with pRPA5, which includes the *nifHDK* operon (Avtges et al. 1983). The striking result is that, whith two exceptions, each cosmid identifies different *Hin*dIII an *Bam*HI fragments, meaning that the different cosmids do not contain neighboring *Eco*RI fragments. One exception is the region containing pRCN101 and pRCN103D, both of which hybridize to a 11.3-kb *Hin*dIII fragment and a 3.05-kb *Bam*HI fragment. Restriction of pRCN101 with *Bam*HI showed a single site at position 5.5 of the insert (Fig. 1A). This result means that the *R. capsulata* fragments of pRCN103D are adjacent and to the right of those of pRCN101 (see Fig. 3).

The second exception involves pRPA8 and pRCN200. Both plasmids identify a 20 kb *Bam*HI fragment. The sizes assigned to the *Eco*RI and *Hin*dIII fragments common to both plasmids are similar. The distribution of *Hin*dIII and *Sal*I sites within the insert of pRCN200 are identical to the sites found in the 5 kb *Eco*RI fragment overlapping pRPA5 (Scolnik and Haselkorn 1984). Therefore the *Eco*RI fragment of pRCN200 is adjacent to the *nif*K gene of pRPA8.

Discussion

Construction of a cosmid library of *R. capsulata* DNA has allowed us to clone a number of genes involved in nitrogen fixation. Cosmid pRCN101 complements the Nif⁻ mutation J43. It contains two *nif* determinants to the left of insertion site 2.8 and at least two *nif* determinants in the 4.8 kb region to the right of 2.8 (Fig. 1A). Cosmid pRCN103D did not complement J62, although recombination of transposons from pRCN103D into the *R. capsulata* chromosome revealed the presence of *nif* genes over at least a 1.45 kb region of the cosmid. Southern hybridization results showed that the *Eco*RI fragments in both these cosmids hybridized to similar chromosomal *Bam*HI and *Hin*dIII fragments, indicating that the *Eco*RI fragments in pRCN101 and pRCN103D are adjacent in the chromosome. The location of the *Bam*HI site in pRCN101 allowed us to map the *Eco*RI fragments from pRCN103D to the right of pRCN101, because the two cosmids hybridized to a similar 3.05-kb chromosomal *Bam*HI fragment. A DNA fragment of that size could extend outside of pRCN101 only to the right of the *Bam*HI site. Also, the *Bam*HI sites within the 5.5-kb *Eco*RI fragment are approximately symmetrically located, which allowed us to place the 1.45-kb *Eco*RI fragment from pRCN103D to the right of the 5.5 kb *Eco*RI fragment.

The resulting restriction map of the pRCN101–

pRCN103D region of the *R. capsulata* chromosome is shown in Fig. 3. This region probably contains the cluster of *nif* genes recently noted by Pühler et al. (1984). Their map of *Eco*RI and *Hin*dIII sites is consistent with that shown in the figure. The two small *Eco*RI fragments to the left of pRCN101 are located there on the basis of their map. Vertical arrows in the figure indicate the locations of Tn*5* insertions in this work or in the study of Pühler et al. (1984). These authors showed the location of only those insertions that resulted in a Nif$^-$ phenotype. We recovered two chromsoomal Tn*5* insertions with a Nif$^+$ phenotype in this region, at 5.8 kb and 14.3 kb on the map in Fig. 3. The first of these occurs in a region saturated with Nif$^-$ insertions. Either it is a fortuitous hit between two operons or it is placed in an intergenic region in a way that allows a promoter in the Tn*5* terminal sequence to transcribe the downstream *nif* genes (Berg et al. 1980). The second Nif$^+$ insertion, at 14.3 kb, may define the right hand boundary of this *nif* region.

Combining the results of Klipp and Pühler with ours, the region of the *R. capsulata* chromosome from 0.8 kb to 14.2 kb on the map in Fig. 3 appears to be filled with *nif* genes. If we allow an average of 1.6 kb per gene (estimated from *Klebsiella* data), the region can accommodate eight *nif* genes. We have shown that the *nifHDK* genes (Avtges et al. 1983) and three *nif* regions, mutated in J61, PA3 and LJ1, map elsewhere. Using *nif*::*lacZ* gene fusions, we have recently shown that the three genes on pRCN102 and the one gene on pRCN200 are *nif* regulatory genes (Kranz and Haselkorn 1985). Therefore the candidates for the pRCN101–pRCN103D cluster are the genes encoding enzymes for the synthesis of Fe–Mo cofactor, for electron transport, and for nitrogenase maturation. In *Klebsiella*, these genes comprise five operons consisting of four, two, two, one and one gene, respectively (Ausubel and Cannon 1980; Brill 1980). In *R. capsulata*, this region has at least three operons, if it is accepted that the Tn*5* insertion at 5.8 kb (Fig. 3) is between operons. Either the region from 0.8 to 2.3 kb in Fig. 3, or the region from 5.0 to 5.7 kb, must be transcribed from a different promoter than the gene mutated in J43. If the J43 gene is transcribed to the right, the region from 0.8 to 2.3 kb must be independently transcribed. If the J43 gene is transcribed to the left, it must have its own promoter because the insertions at 5.0 and 5.7 kb do not interfere with cosmid pRCN101 complementation of J43. None of our Tn*5* chromosomal insertions in this region (Fig. 3) mutates a *nif* regulatory gene, since all are capable of activating a *nifH*::*lac* fusion (Kranz and Haselkorn 1985).

Not all of the non-regulatory, non-nitrogenase *nif* genes are in this cluster. The gene mutated in PA3 is unlinked to this cluster (Fig. 1C) and it is not a regulatory gene (Kranz and Haselkorn 1985). Pühler et al. (1984) identified one more Tn*5* insertion to the left of the 7.7-kb *Hin*dIII fragment (Fig. 3). They do not show the restriction map for the region of this insertion so we cannot relate it to our other cosmid clones. The gene or region mutated in PA3 is rather small, since the Tn*5* insertions that prevent complementation define a maximum size of 1.1 kb. Our inability to recover chromosomal insertions of Tn*5* in the vicinity of PA3 suggests that the region contains genes essential for growth on minimal medium with ammonia (RCVB).

The final cosmid to be discussed, pRCN102, complements the regulatory mutation J61. Tn*5* insertions map the J61 complementing activity to a region of maximum size 2.3 kb (Fig. 1D). Chromosomal insertions of Tn*5* identify a second *nif* region to the right of the J61 mutation, whose maximum size is 1.2 kb. It appears to be transcribed independently of the J61 gene because a Tn*5* insertion at 2.3 kb, between the two genes, does not prevent complementation of J61 (Fig. 1D). A third *nif* gene on pRCN102 was located by Tn*5* 4.3. This region is also transcribed independently of the first two regions because a Tn*5* at 3.5 yields a Nif$^+$ phenotype when recombined into the chromosome.

Hybridization of nick-translated clone probes to digests of chromosomal DNA showed that, with the exception of pRCN101–pRCN103D, none of the cosmids overlap or are immediate neighbors (Table 1). Mutants J43 (group I) and J62 (group II) of Wall and Braddock (1984) are in the cluster shown in Fig. 3. The two mutations are separated by at least 5.3 kb, which explains why they are unlinked by GTA analysis (Wall and Braddock 1984). Their mutants J56, J57 and J60 (group IV) were previously shown by us to be in the *nifHDK* gene cluster (Avtges et al. 1983). Mutant LJ1 (group VI) was shown to be unlinked to other *nif* genes by GTA transduction (Wall et al. 1984). However, we located that gene downstream of *nifHDK* in the next *Eco*RI fragment. The orientation of this gene is the same as that of *nifHDK* (Kranz and Haselkorn 1985). Mutant J61 (group III) is in a separate cluster.

We can summarize the mapping results as follows: the structural genes for nitrogenase components (*nifHDK*) are in one operon (Avtges et al. 1983), linked to the regulatory gene mutated in LJ1. Possibly as many as eight *nif* genes, in at least three operons, are located within a 14 kb region (Fig. 3). An additional non-regulatory *nif* locus, mutated in PA3, is in a third region (Fig. 1C). A regulatory *nif* gene, mutated in J61, is located in a fourth region which contains at least two more *nif* genes. We estimate that we can now account for approximately 16 *nif* genes in four distinct locations in the *R. capsulata* chromosome. On four out of four *Eco*RI fragments in which Tn*5* inserts were recombined into the chromosome (pRCN101, pRCN103D, pRCN106 and pRCN102) genes or regions yielding Nif$^+$ phenotypes were located. Thus, the *nif* operons of *R. capsulata* appear to be dispersed with non-*nif* DNA separating the local clusters. Simply adding the sizes of the non-overlapping *Hin*dIII fragments in Table 1 indicates that the *nif* genes of *R. capsulata* will extend over at least 70 kb, corresponding to more than twice the distance needed for the *nif* genes of *Klebsiella*.

The *nif* genes isolated in this work and the Tn*5* directed *nif* mutants described here can now be used for studies on the detailed analysis of individual genes. Biochemical characterization of the independent, genetically defined mutants is also now possible. Previous work by Wall et al. (1984) on LJ1 has suggested that this mutant may be regulatory. We have recently shown that *nif* mutants LJ1, AH1, AH3, J61 and the Tn*5* mutagenized *nif* genes on pRCN102 (Tn*5* 1.1, 2.5, 2.7 and 4.3) are all regulatory mutants, using an *R. capsulata nif*H::*lac*Z fusion (Kranz and Haselkorn 1985).

Acknowledgments. We are grateful to Judy Wall and Ayala Hochman for providing Nif$^-$ mutants and to Pablo Scolnik and Helen Revel for valuable advice. This work was supported by research grants from the National Institutes of Health (GM21823) and the

USDA/SEA through the Competitive Research Grants Office (82-CRCR-1-1056) and by a predoctoral traineeship from NIH to P.A. (GM 07197), and a postdoctoral fellowship from NIH to R.G.K. (GM 10466).

References

Ausubel FM, Cannon FC (1980) Molecular genetic analysis of *Klebsiella pneumoniae* nitrogen fixation (*nif*) genes. Cold Spring Harbor Symp Quant Biol 45:487–497

Avtges P, Scolnik PA, Haselkorn R (1983) Genetic and physical map of the structural genes (*nifHDK*) coding for the nitrogenase complex of *Rhodopseudomonas capsulata*. J Bacteriol 156:251–256

Berg DE, Weiss A, Crossland L (1980) Polarity of Tn*5* insertions in *Escherichia coli*. J Bacteriol 142:439–446

Boyer HW, Rouland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41:459–472

Brill W (1980) Biochemical genetics of nitrogen fixation. Microbiol Rev 44:449–467

Castilho BA, Olfson P, Casadaban MT (1984) Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J Bacteriol 158:488–495

Ditta G, Stanfield S, Corbin D, Helinski D (1980) Broad host range cloning system for gram negative bacteria: contruction of a gene bank of *Rhizobium meliloti*. Proc Natl Acac Sci USA 77:7347–7354

Enquist L, Sternberg N (1979) In vitro packaging of *λdam* vectors and their use in cloning DNA fragments. Meth Enzymol 68:281–298

Friedman AM, Long SR, Brown SE, Buikema WJ, Ausubel FM (1982) Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene 18:289–296

Hirsch PR, Beringer JE (1984) A physical map of pPH1J1 and pJB4J1. Plasmid 12:139–141

Johansson BC, Gest H (1977) Adenylation/deadenylation control of the glutamine synthetase of *Rhodopseudomonas capsulata*. Eur J Biochem 81:365–371

Kranz R, Haselkorn R (1985) Characterization of *nif* regulatory genes in *Rhodopseudomonas capsulata* using *lac* fusions. Gene, in press

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 433

Pühler A, Aguilar MO, Hynes M, Müller P, Klipp W, Priefer U, Simon R, Weber G (1984) Advances in the genetics of free-living and symbiotic nitrogen fixing bacteria. In: Veeger C, Newton WE (eds) Advances in nitrogen fixation research. Nijhoff/Junk, The Hague, pp 609–619

Ruvkun GB, Ausubel FM (1981) A general method for site-directed mutagenesis in prokaryotes. Nature 289:85–89

Scolnik PA, Haselkorn R (1984) Activation of extra copies of genes coding for nitrogenase in *Rhodopseudomonas capsulata*. Nature 307:289–292

Scolnik PA, Virosco J, Haselkorn R (1983) The wild type gene for glutamine synthetase restores ammonia control of nitrogen fixation to Gln$^-$(*glnA*) mutants of *Rhodopseudomonas capsulata*. J Bacteriol 155:180–185

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Wall JD, Braddock K (1984) Genetic mapping of *nif* genes in *Rhodopseudomonas capsulata*. J Bacteriol 158:404–410

Wall JD, Love J, Quinn SP (1984) Spontaneous Nif$^-$ mutants of *Rhodopseudomonas capsulata*. J Bacteriol 159:652–657

Yen HC, Marrs B (1976) Map of genes of carotenoid and bacteriochlorophyll biosyntheesis in *Rhodopseudomonas capsulata*. J Bacteriol 126:619–629

Communicated by P. Tiollais

Received August 28, 1985