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Fine-Structure Mapping and Complementation Analysis of *nif* (Nitrogen Fixation) Genes in *Klebsiella pneumoniae*

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Four hundred and eighty-nine independent Nif$^-$ strains containing 260 point, 130 Mu-induced, and 99 deletion mutations in *nif* in the *Klebsiella pneumoniae* chromosome were isolated. Three hundred and ninety insertion and point mutations were mapped with Mu-induced deletions carried on 44 plasmids derived from pTM4010, a recombinant R factor containing the *his-nif* region of *K. pneumoniae*. The 99 chromosomal deletions in the *nif* region were mapped with 69 derivatives of pTM4010 carrying insertion and point mutations in *nif*. Complementation analysis between 84 derivatives of pTM4010 carrying *nif* mutations and Rec$^-$ derivatives of the 390 Nif$^-$ mutants identified 14 genes. The *nif* mutations were ordered into 49 deletion groups with a gene order of *his... nifQBALFMVSNEKDHJ*. Complementation analysis of Mu-induced, amber, frameshift, and deletion mutations indicates there are five polycistronic and two monocistronic operons: *nifQ nifB*, *nifA nifL*, *nifF*, *nifM nifV nifS*, *nifN nifE*, *nifK nifD nifH*, and *nifJ*. Transcription is from right to left in all polycistronic operons.

A complete understanding of N_2 fixation requires a knowledge of the number of genes involved, the organization and control of these genes, and the identification of the function of each gene. The genetics of N_2 fixation has been studied primarily in *Klebsiella pneumoniae*, where the *nif* genes have been shown to map near *his* by P1 transduction (36) and by conjugation (13). Strains containing point mutations (12, 20, 22, 33, 35, 36) and a few Mu-induced mutations (29) in *nif* have been isolated. Recent complementation analyses described seven *nif* cistrons (12), and transductional analyses indicated the possible existence of two additional cistrons (22). Mapping of *nif* mutations has been accomplished primarily by P1 transduction by measuring the frequency of cotransduction with *his* (20, 22, 35, 36) and by ordering mutations by three-factor crosses using *his* as the third marker (20, 22, 33).

The bacteriophage Mu is a powerful genetic tool for isolating polar, nonreverting mutations due to prophage insertion and also for the generation of deletion mutations (19). Recently Mu has been used to generate deletions in the *his-nif* region of *K. pneumoniae* (4) and also in the plasmid pTM4010 (24). The plasmid pTM4010 is a derivative of pRD1, a recombinant R factor containing the *K. pneumoniae his-nif* region which no longer confers resistance to P1 and Mu (11, 24). This derivative enhances the usefulness of the plasmid by permitting use of Mu for genetic manipulations and the use of P1 for transducing mutations in strains carrying the plasmid. Derivatives of pTM4010 can be constructed by transducing defined *nif* mutations into the plasmid which can then be used for merodiploid analysis of *nif* functions.

We have isolated several hundred Nif$^-$ strains containing point mutations, Mu insertions, and Mu-induced deletions. Plasmids containing *nif* mutations were used in complementation analyses to determine the number of *nif* genes. Polar mutations were used to determine the number of *nif* transcripts and their direction of transcription. Mutations in *nif* were mapped by deletion analyses to obtain a detailed map of the *nif* genes.

(Part of this work has been presented previously [T. MacNeil, G. P. Roberts, D. MacNeil, M. A. Supiano, and W. J. Brill, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, K31, p. 131].)

MATERIALS AND METHODS

Media. Recipes for LC medium, P1 diluent, and B diluent (4) and for soft agar and minimal medium have been described (24). Solid media contained 15 g of agar (Difco Laboratories, Detroit, Mich.) per liter except N-free media, which contained 13 g of purified agar (Difco) per liter. Amino acids were added to 20 μg/ml when required. 6-Cyanopurine (CP) plates have been

described (23). When necessary, filter-sterilized antibiotics were added to the medium to the following concentrations: tetracycline (Lederle, Pearl River, N.Y.) at 20 μg/ml, kanamycin (Sigma Chemical Co., St. Louis, Mo.) at 25 μg/ml, and chloramphenicol (Sigma Chemical Co.) at 12.5 μg/ml. Diethyl sulfate (DES) was obtained from Eastman Kodak Co., Rochester, N.Y. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was obtained from Aldrich Chemical Co., Milwaukee, Wis. Hydroxylamine hydrochloride (HA) was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. ICR191F was a gift from H. Creech, Institute for Cancer Research, Fox Chase, Philadelphia, Pa.

Bacterial strains. Strains of *K. pneumoniae* and *E. coli* K-12 used in this work are listed in Table 1. UN727 and UN729 are Mu-sensitive derivatives of UN isolated as mutants resistant to a *K. pneumoniae* virulent phage (4). UN729 is moderately Mu sensitive and P1*kc* resistant, but P1*kc h1* sensitive. UN727 is Mu sensitive and P1*kc h2* sensitive, but resistant to P1*kc* and P1*kc h1*. UN1007 is a heat-resistant, Nif$^+$ derivative of UN878.

Bacteriophage strains. P1*kc* was obtained from W. H. McClain. P1*kc h1* is a spontaneous host range mutant of P1*kc*, isolated by plating P1*kc* on UN729, which grows on UN and UN729 but not on UN727. P1*kc h2* is a spontaneous host range mutant of P1*kc*, isolated by plating P1*kc* on UN727, which grows well on UN727 but poorly on UN and UN729. P1CM*clr100* (31) and Mu *cts61* and Mu *c25* have been described (1, 18). Mu lysates were prepared as described previously (4).

Isolation of Nif$^-$ point and insertion mutants. Strains containing point mutations or Mu insertions in *nif* were isolated after penicillin enrichment of mutagenized cultures, except that HA-induced and some DES-induced mutants were isolated without penicillin enrichment. After mutagenesis, cells were grown to stationary phase in minimal medium with 0.2% ammonium acetate. They were diluted in fresh medium and grown to 3 to 5 × 10^8 cells/ml. Cells were washed and resuspended in N-free medium at 5 × 10^7 cells/ml and flushed with N_2. They were grown on N_2 for 6 h at 30°C. Penicillin G was added to 10,000 U/ml, and the culture was incubated for another 6 h. Cells were collected by filtration, washed with distilled water, resuspended, and grown overnight in minimal medium with NH_4^+. The culture was diluted and

TABLE 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype[a]	Source
Strain		
UN	*K. pneumoniae* M5a1 (wild type)	P. W. Wilson
UN209	*hisD4226*	24
UN562	*pro-4001* Δ(*rfb gnd his nif*)	Δ16, of ref. 34
UN727	*rfb-4002*	
UN729	*rfb-4001*	4
UN878	*rfb-4001 his-4201*::Mu *cts61*	4
UN976	*pro-4001* Δ(*rfb gnd his nif*) (pTM4010)	
UN1007	*rfb-4001* Δ*his-4208*	
UN1287	*rpoB4001*	
UN1288	*rpoB4001 recA56 srl-300*::Tn*10*	
UN1289	UN1288 (P1CM*clr100*)	
UN1290	*hisD4226 recA56 srl-300*::Tn*10*	
UN1291	UN1290 (P1CM*c1r100*)	
UN1516	*pro-4001* Δ(*rfb gnd his nif*) (pTM4041)	
UN1549	Δ(*his-nif*)*4648*	Eductant of ref. 34
UN1963	*pro-4001 arg-4001* Δ(*rfb gnd his nif*) (pTM4041)	
CK263	*nifA2263 hisD2 hsdR1 rpsL4*	12, 22
CK265	*nifL2265 hisD2 hsdR1 rpsL4*	12, 22
CK296	*nifE2296 hisD2 hsdR1 rpsL4*	12, 22
CK319	*nifJ2319 hisD2 hsdR1 rpsL4*	12, 22
E. coli K-12		
JC10240	Hfr P045 *srlA300*::Tn*10 recA56 thr-300 ilv-318 thi-1 rpsE300*	L. Csonka
MH812	*hsdM hsdR thr leu met lac supD*	921 of W. Arber
N100(RP4 Tet^s(Am) $\beta G\beta^{Mu}$ SuA1P2-1)	*rpsL recA* (*amp*$^+$ *tet*$^+$ *kan*$^+$ SuA1P2-1)	J. Schell (14)
N100(RP4 Tet^s(Am) $\beta G\beta^{Mu}$ Sul-3)	*rpsL recA* (*amp*$^+$ *tet*$^+$ *kan*$^+$ Sul-3)	J. Schell
Plasmid		
pTM4010	*amp*$^+$ *tet*$^+$ *kan*$^+$ *gnd*$^+$ *his*$^+$ *nif*$^+$ *shi*$^+$	24
pTM4014	*amp*$^+$ *tet*$^+$ *kan*$^+$ *gnd*$^+$ *his*$^+$ *nif*$^+$ *shi*$^+$ (Mu *cts Cam4005*)	24
pTM4041	*amp*$^+$ *tet*$^+$ *kan*$^+$ *gnd*$^+$ *hisD4209 nif*$^+$ *shi*$^+$	

[a] Abbreviations are those described by Bachmann et al. (5) and Novick et al. (28).

plated on minimal medium without ammonium acetate. Plates were incubated anaerobically at 30°C for 3 to 4 days. Tiny colonies were picked as possible Nif⁻ mutants, purified, and retested on N-free medium.

NG mutagenesis was performed as described (2). NG-induced mutations, *nif-4001* through *nif-4282*, were isolated from UN by R. T. St. John (Ph.D. thesis, University of Wisconsin, Madison 1973). ICR191F-induced mutations *nif-4768* through *nif-4810* and *nif-4940* were isolated from UN as described (27). HA-induced mutations *nif-4602*, *nif-4609* through *nif-4621*, and *nif-4625* through *nif-4645* were isolated after transduction of UN209 to His⁺ by P1*kc* grown on UN and mutagenized by HA (25). Transductants were tested on N-free medium to identify Nif⁻ mutants.

Cultures on UN729 and UN727 lysogenic for Mu *cts61* were isolated as described (4). When the frequency of lysogens in a culture was below 10%, infection of the culture with Mu *c25*, a clear mutant, was used to enrich the population for lysogens. Nif⁻ strains containing Mu-induced *nif-5026* through *nif-5239* and *nif-4985* were isolated from UN727. Mu-induced *nif* mutations *nif-4405* through *nif-4483* were isolated from UN729 excluding *nif-4428*, *nif-4430*, and *nif-4459*. These are three spontaneous *nif* mutations in strains derived from UN729.

All other *nif* point mutations listed in Fig. 1 are DES-induced mutations in strains derived from UN except *nif-4591* through *nif-4601* and *nif-4356* through *nif-4368* which are from UN1007. DES mutagenesis was performed as described (32). Strains containing DES-induced mutations *nif-4373* through *nif-4403* were isolated without penicillin enrichment as mutants with altered color on CP plates (23).

Not all Nif⁻ strains were tested for reversion to Nif⁺. It is possible that some mutations described as point mutations may be small insertions or deletions within a gene. Assay of Nif activity by acetylene reduction has been described (6, 23).

Isolation of strains with Nif⁻ Mu-induced deletions. Strains with Mu-induced *nif* deletions from Mu lysogens of UN729 or UN727 were isolated among survivors of heat induction of strains with Mu insertions in *his* or *nif* as described (4, 23). In Fig. 1 the *nif* deletion mutations Δ*nif-4331*, Δ*nif-4332* through Δ*nif-4338*, Δ*nif-4340*, Δ*nif-4342* through Δ*nif-4352*, Δ*nif-4972*, Δ*nif-4973*, and Δ*nif-4974* through Δ*nif-4981* are contained in the plasmids pTM4011, pTM4015 through pTM4021, pTM4023, pTM4025 through pTM4035, pTM4039, pTM4040, and pTM4086 through pTM4093, respectively (24).

Construction of plasmids with *nif* mutations. Phage P1*kc*, P1*kc h1*, or P1*kc h2* was grown on strains with mutations by the confluent lysis method (4). Mutations were transduced into plasmid pTM4041 by transducing strain UN1516 or UN1963 to His⁺. Both of these strains contain a chromosomal deletion of the *rfb gnd his nif* region. Transductions were performed as described (38). His⁺ transductants were tested for cotransduction of the *nif* mutation by assaying growth on N-free medium. In addition, if the mutation being transduced into the plasmid was Mu induced, phage release was scored on strain MH812 (4).

Mapping of *nif* mutations. Chromosomal deletions were mapped by mating plasmids containing *nif* point mutations or Mu insertions into all strains containing chromosomal deletions. Mu-induced or point mutations in the chromosome were mapped by crossing strains with these mutations with strains carrying plasmids having partial deletions of *nif*. For these crosses, 0.1 ml of an overnight culture of a Nif⁻ recipient strain was spread over the surface of an LC plate, and then 0.05-ml amounts of five different donor strains were spotted on the lawn of recipient cells. These mating plates, together with control matings using a donor containing a plasmid totally deleted for *nif* and unmated controls, were incubated overnight at 30°C and replicated to N-free medium made with purified agar. These plates were incubated anaerobically for 5 days at 30°C. For mapping leaky mutations, selective plates of N-free medium with kanamycin were incubated at 35°C. The exconjugants were scored as Nif⁺ if growth occurred in the mixed patch of donor and recipient on N-free medium but not in control matings or unmated controls. A Nif⁻ phenotype was scored if no growth occurred with Nif⁻ recipients but growth did occur with His⁻ Nif⁺ recipients.

Construction of Rec⁻ *K. pneumoniae* strains. P1CM*clr100* lysates of the Tet^r Rec⁻ strain UN1291 were prepared as described (31). All lysates were made by induction of P1CM lysogens because P1*kc* grows very poorly on Rec⁻ strains. All strains with *nif* mutations were transduced to Tet^r by mixing 0.1 ml of lysate (2×10^8 plaque-forming units/ml) with 2×10^8 cells and plating the mixture on LC plates with 20 μg of tetracycline per ml. Plates were incubated for 1 to 2 days at 30°C. Tet^r transductants and Rec⁺ and Rec⁻ control strains were patched onto LC plates. After overnight growth, they were replicated to LC plates, and these plates were irradiated for 50 s at 75 cm by a Westinghouse UV sterile lamp, model 782L-30. Plates were incubated at 30°C in the dark for 18 h. Rec⁺ UV-resistant strains formed a confluent patch of growth, whereas Rec⁻ UV-sensitive strains produced 0 to 10 single colonies.

Complementation of *nif* mutations. Complementation tests were performed as described above for mapping mutations with two modifications. Rec⁻ recipients were used, and 10 μl of each of 25 donors was spotted on the lawn of recipient cells.

RESULTS

Isolation of strains with insertion and point mutations in *nif*. We isolated 390 mutants with independent chromosomal *nif* insertion and point mutations from 53 cultures treated with a variety of mutagens. Mutants from the same culture were distinguished as independent by virtue of their map position, reversion by different mutagens, level of in vivo acetylene reduction, and phenotype on two-dimensional polyacrylamide gel electrophoresis (30). Different mutagens were used to induce *nif* mutations to assure that all *nif* genes would be mutagenized and to isolate polar and nonpolar mutations. Phage Mu and ICR191F cause polar mutations by insertion and induction of frame-

POINT MUTATIONS

2263 4070 4031
4176 4105 4066
4227 4234
4273 4430
4379 4605
4106 4381 4607
4064 4378 4383 4625
4188 4402 4395 4692 4027
4672 4603 4400 4718 4093
4679 4613 4403 4734 4230
4691 4683 4598 4737 4690
4780 4721 4708 4611 4746 4733 5499
4388 4743 4739 4682 4758 4750 4092
5406 4620 4748 4766 4783 4765 4756 4459 4277
4969 4681 4786 4772 4790 4806 4767 4940 4282 4074 4955
4970 4796 4814 4600 4805 4791 4373 4816 4820 4944 4942 4198 4961
5251 4398 4809 4968 4674 4949 4808 4602 2265 4829 4946 4962 4945 4967 4389 4618 4966

4413
4418
4438
5051

MU INSERTIONS

5044 4445 5061
4422 5049 5174 4425 4423 4417 5065
4427 4478 5082 5177 5176 5047 4471 4457 5074
4477 5037 5224 5182 5229 5050 5073 5038 5048 5077
5027 5026 4433 4985 5178 5227 5036 5226 5221 5234 5183 5084 5041 5083 5085
5031 5030 4444 4463 4408 5232 5228 5238 5181 5233 5237 5239 5184 5087 5057 5098 5067 5101

his *nifQ* *nifB* *nifA* *nifL* *nifF* *nifM* *nifV* *nifS* *nifN*

DELETIONS

4343,4344,4347,4932
4349
4926,5253
4534,4553
4973
4832
4890,5000
5001
4975
4331
4529
4340,4872,4980
4979
4934
4333
4338
4972
4332
4842

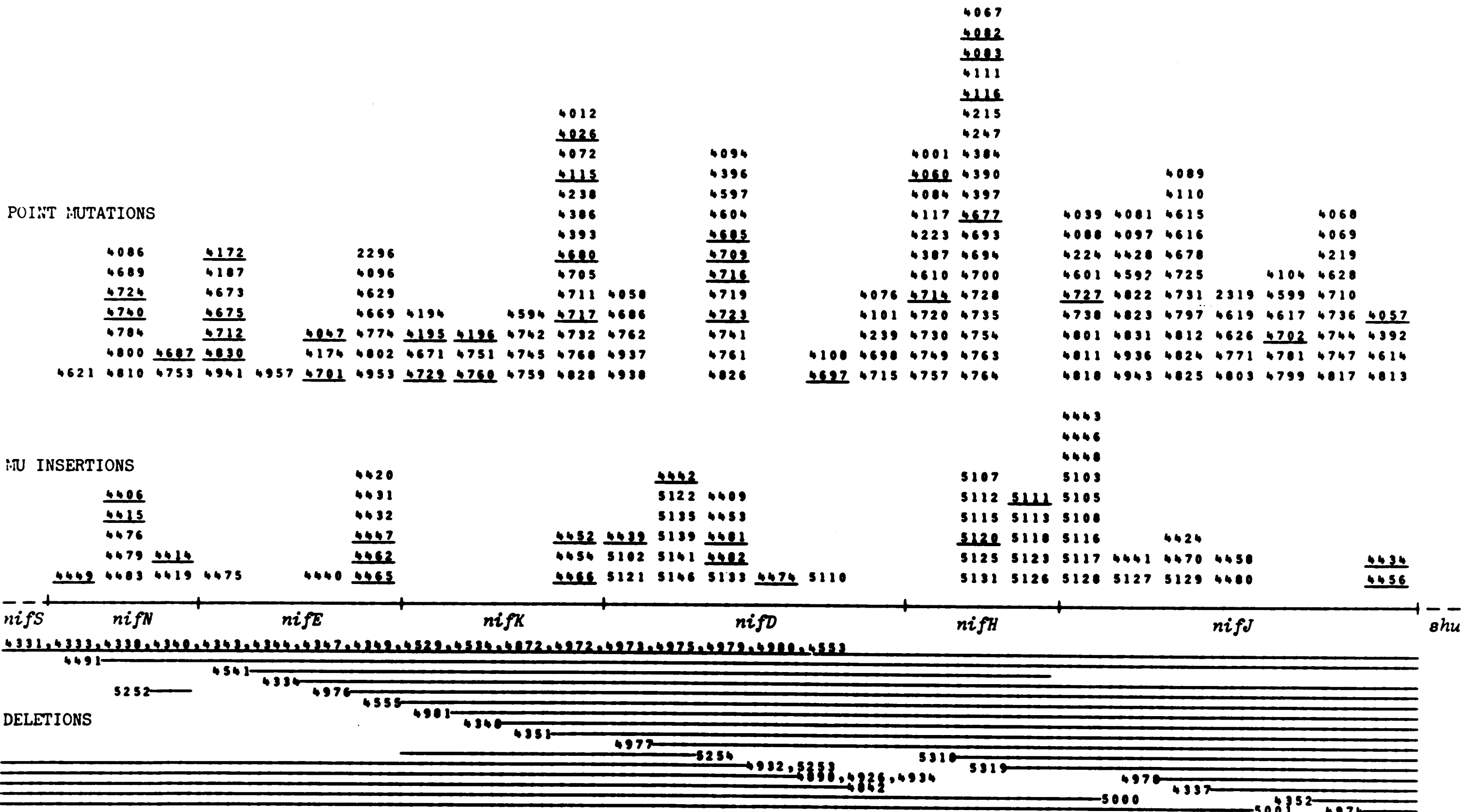

FIG. 1. *Complementation groups and mapping of point mutations and Mu-induced mutations in nif. Complementation groups are designated by the genes nifQ through nifJ, and mutations in each complementation group are listed above the appropriate nif gene. nifL mutations are nifA in complementation tests. Mutations that were transduced into pTM4041 and used in complementation analyses are underlined. nif deletions in derivatives of pTM4010 are represented by solid lines, and the allele number of each deletion is listed at the end of the deletion. Allele numbers which are above the first deletion on each portion of the map indicate deletions which extend through either the nifQ-nifN or the nifE-nifJ region.*

shift mutations, respectively (9, 21, 26). DES, NG, and HA induce predominantly nonpolar point mutations (27). One hundred fifty-six DES-induced, 130 Mu-induced, 62 NG-induced, 23 ICR191F-induced, 16 HA-induced, and 3 spontaneous *nif* mutations were obtained. All of the mutants were restored to Nif$^+$ by introducing the His$^+$ Nif$^+$ plasmid pTM4010, indicating that all of the mutations are in the *his-nif* region and are recessive. In addition to the 390 mutants described above, 8 Nif$^-$ strains which could not be restored to Nif$^+$ by introducing pTM4010 were isolated by procedures described above. These strains were unable to utilize a variety of organic N sources, suggesting that the mutations were not *nif* specific.

To aid in the mapping and complementation studies of the *nif* mutations, 84 Mu insertion and point mutations were transduced into the plasmid pTM4041 by transducing either strain UN1516 or UN1963 to His$^+$ and scoring for Nif$^-$. The 84 mutations transduced into pTM4041 are indicated by an underscore in Fig. 1.

Isolation of strains with *nif* deletions. Strains with *nif* deletions were isolated from Mu lysogens. Some heat-resistant mutants derived from Mu *cts* lysogens contain deletions of the prophage and neighboring bacterial genes (18). In these strains, the deletion removes at least the *c* gene immunity end of the Mu prophage and extends for variable lengths into adjacent bacterial genes. Bachhuber et al. obtained strains containing deletions extending into *nif* by isolating heat-resistant strains unable to release Mu from lysogens with Mu insertions in *his* (4). We have used this technique to obtain a variety of His$^+$ and His$^-$ strains with *nif* deletions in the *K. pneumoniae* chromosome and in a derivative of plasmid pTM4010.

Strains with chromosomal *nif* deletions were isolated and recognized in a variety of ways. In addition to the five strains described by Bachhuber et al. (4), four strains with *his-nif* deletion were isolated from UN878 by scoring survivors of heat selections for their Nif$^-$ phenotype. Strains containing *his-nif* deletions also were isolated from UN878 by directly plating survivors of heat selections on CP plates and isolating white colonies. MacNeil and Brill (23) have shown that 6-CP is an indicator of *nifA* gene expression. Nif$^+$ and most Nif$^-$ strains form purple colonies on CP plates, whereas all strains with point mutations or deletions into *nifA*, and some strains with deletions of other *nif* regions, form white colonies. Four mutants with *nif* deletions were isolated from UN878 as white colonies on CP plates. CP plates also were used to screen survivors of heat selections from 32 strains with Mu insertions in *nif*. Twelve mutants with His$^-$ Nif$^-$ deletions and 36 mutants with His$^+$ Nif$^-$ deletions were isolated as white colonies on CP plates with and without histidine, respectively (23). Fifteen strains with His$^+$ Nif$^-$ deletions were isolated from 11 strains with Mu insertions in *nif* by screening survivors of heat selections for deletion of *nif* genes adjacent to the Mu insertion. Finally, *his-nif* deletions in 23 strains were identified by scoring for survivors of heat selections from 16 strains, with Mu insertions in *nif*, for His$^-$. Using these procedures, we have isolated the 99 strains with *nif* deletions shown in Fig. 2. These were isolated from one strain with a Mu insertion in *his* and 54 strains with Mu insertions in *nif*.

Strains containing plasmids with *nif* deletions were isolated in a variety of ways. Several of the deletions shown in Fig. 1 were obtained from heat induction of strains carrying the plasmid pTM4014, which has a Mu insertion to the right of *nifJ*. Deletion mutations Δ*nif-4333* through Δ*nif-4352* and Δ*nif-4972* through Δ*nif-4981* are the results of such heat inductions and were previously mapped against 26 *nif* mutations (24). Two strains isolated with Mu insertions in pTM4010 also contained Δ*nif-4331* and Δ*nif-4332* and were described recently as plasmids pTM4011 and pTM4015, respectively (24). White colonies on CP plates were isolated after heat induction of two strains with derivatives of pTM4010 which have Mu insertions in *nifH*. Plasmids with Δ*nif-5319* and Δ*nif-5318* are derived from plasmids with *nifH5111*::Mu *cts61* and *nifH5120*::Mu *cts61*, respectively. The additional deletions shown in Fig. 1 were constructed by transducing chromosomal deletions into the plasmid pTM4041. Phage P1 was grown on His$^+$ strains with a deletion in *nif* and used to transduce UN1516 or UN1963, both of which contain the plasmid pTM4041 that has a point mutation in *his*, to His$^+$. Transductants were scored for cotransduction of the *nif* deletion. The deletions Δ*nif-5252*, Δ*nif-5253*, and Δ*nif-5254* were spontaneous *nif* deletions isolated among His$^+$ transductants of UN1963.

Mapping. Mapping of chromosomal point mutations and Mu insertions was performed by crossing these mutants with strains containing *nif* deletion plasmids by conjugation. Matings between strains carrying Nif$^-$ plasmids and Nif$^-$ recipients were done on LC medium, and the bacteria were then replicated to N-free medium to detect Nif$^+$ recombinants. The plasmid transfers at a frequency of 10^{-1} per donor cell (11) so that a large number of mating pairs are formed. A positive result was scored if the number of Nif$^+$ recombinants was significantly higher than

FIG. 2. *Map of Mu-induced nif deletions in the K. pneumoniae chromosome. Chromosomal deletions were mapped by derivatives of pTM4010 bearing mutations listed above each nif gene. Deleted DNA is indicated by solid lines, and the allele number of each deletion is given at the end point of the deletion. nifL is not designated on this map because no nifL mutations were used to map these deletions. Allele numbers which are above the top deletions on each portion of the map indicate deletions which extend through his-nifN, nifQ-nifN, or nifE-nifJ.*

the number of Nif^+ colonies in control matings and on unmated controls (usually zero to five). With this technique we saw greater than 1,000 Nif^+ recombinants between two mutations, Δ*nifJ4974* and *nifJ4081*, within a single gene. All mutations were mapped several times, and reproducible results were obtained. It is possible that mutations which actually mapped outside but very near the end point of a deletion were scored as mapping under the deletion due to the presence of very few (zero to five) recombinants. All plasmids transferred well in crosses with UN209 when His^+ exconjugants were selected. None of the plasmids carried a dominant *nif* mutation.

Forty-four of 260 (17%) strains containing point mutations and 4 of 130 (3%) strains containing Mu-induced mutations were leaky on N-free medium. These mutations were mapped by selecting Nif^+ recombinants on N-free medium with kanamycin at 35 instead of 30°C. The addition of kanamycin prevents growth of the lawn of the leaky Nif^- recipient, allowing much easier scoring of Nif^+ recombinants on the section of the plate containing the mated pairs. Nif is expressed at a lower level at 35 than at 30°C, so incubation of selective plates at this temperature reduces residual activity in leaky Nif^- mutants. Mapping of mutations in leaky and nonleaky Nif^- strains has established the extent of the 44 plasmid *nif* deletions and ordered the 390 insertion and point mutations into 43 deletion groups as shown in Fig. 1.

Strains which have chromosomal *nif* deletions were crossed with strains containing the Nif^- derivatives of pTM4010. The 69 plasmids with *nif* Mu insertions and point mutations were used to establish the extent of the chromosomal deletions and order those plasmid mutations as shown in Fig. 2. *nifL* is not designated on Fig. 2 because no *nifL* mutations were used to map chromosomal deletions. The mapping results of plasmids with *nif* deletions crossed with chromosomal mutations (Fig. 1) and plasmids with mutations crossed with chromosomal deletions (Fig. 2) gave the same order of *nif* genes. Moreover, many mutations in the same deletion group in Fig. 1 were further ordered by the mapping shown in Fig. 2.

Construction of Rec^- Nif^- strains. A complementation test determines whether two mutations are in the same gene. For this test, a partially diploid strain is constructed with two mutations in the *trans* arrangement. If the wild-type phenotype is restored, the two mutations are assumed to be in different cistrons. For nonpolar point mutations, if the mutant phenotype prevails, the two mutations are in the same cistron. Such a complementation test cannot be performed with *K. pneumoniae* Nif^- mutants and Nif^- plasmids by assaying growth on N-free medium because recombination will occur in the partial diploid restoring the Nif^+ phenotype. However, if the diploid strain is recombination deficient (Rec^-), Nif^+ recombinants will not be generated. To rapidly isolate Rec^- derivatives of all Nif^- mutants, we took advantage of a specially constructed Hfr strain of *E. coli*, JC10240. This strain carries a Tn*10* (Tet^r) insertion in *srl* (sorbitol utilization) which is P1 cotransducible with the *recA56* mutation. JC10240 was mated to rifampin-resistant strain UN1287. A hybrid exconjugant UN1288 was isolated that was Tet^r, Srl^-, UV sensitive, anf Rif^r. P1 lysates were made by induction of a P1CM*clr100* lysogen of this strain (UN1289) and used to transduce UN209 to Tet^r. Transductants were Srl^- and UV sensitive and could not be transduced to His^+, indicating that the strain was recombination deficient. A P1CM*clr100* lysogen of UN1290 (strain UN1291) was isolated, and lysates made from this strain were used to transduce all chromosomal Nif^- strains to Tet^r and Rec^-. Between 50 and 100% of the Tet^r colonies were UV sensitive and Rec^-, whereas greater than 95% of the Tet^r Srl^- colonies were UV sensitive and Rec^-, presumably because recombination between the *E. coli recA srl* region and the *K. pneumoniae rec srl* region was greatly reduced due to nonhomology. The Tet^r Srl^+ strains included spontaneous Tet^r colonies and may have included some strains in which Tn*10* had transposed during the transduction.

To compare the level of recombination in our Rec^+ and Rec^- strains, the plasmid containing *nifJ4057* was mated in Rec^+ and Rec^- strains containing *nifJ4727* and *nifJ4738*, and the diploid was purified. The titer of Nif^+ recombinants in overnight cultures of the four partial diploids was determined. In both cases the number of Nif^+ recombinants from Rec^- heterozygotes was 5×10^{-5} lower than that obtained from Rec^+ heterozygotes. A similar reduction in the number of recombinants (10^{-6}) was observed by Guyer and Clark in Hfr matings to F^- recipients with the *recA56* mutation (17).

Complementation analysis. Eighty-four plasmids containing *nif* point mutations and Mu-induced mutations were mated into Rec^- derivatives of all Nif^- mutants. Matings were done on LC plates which were replicated to N-free medium to determine whether the partial heterozygote was Nif^+ or Nif^-. Confluent growth of the heterozygote was scored as positive, and growth no better than that in control matings (matings into a strain with a total *nif* deletion)

and that of unmated controls was scored as negative. When mutations failed to complement each other, they were assigned to the same complementation group. Polar mutations were assigned to a cistron based on which groups they failed to complement and their map position. Whenever a *nif* allele complemented all other alleles tested, it was transduced into the plasmid and crossed to all possible recipients. In this way additional complementation groups were identified. Over 40,000 partial diploids were constructed with the 390 point mutations and Mu insertions in *nif*. These mutations were assigned to 13 complementation groups as shown in Fig. 1. Complementation analysis was consistent with results of deletion mapping, since all mutations in each complementation group mapped adjacent to each other. Several deletion groups were subdivided by complementation analysis to order the chromosomal mutations into 49 groups.

In general, all point mutations in a gene were unable to complement all other mutations tested in that cistron, but were able to fully complement nonpolar mutations in different cistrons. However, mutations in *nifH* on the plasmid frequently gave only weak complementation with mutations in all of the other groups. This may indicate the presence of inactive H protein competing with functional H protein to impair the ability of a cell to fix N_2. Since this phenomenon was observed only when the *nifH* mutation is on the plasmid, it may reflect a greater proportion of the mutant plasmid protein compared with chromosome protein.

Two mutations in *nifA*, *nifA4395* and *nifA4682*, failed to complement mutations in *nifH* as well as in *nifA*. The results of mapping with plasmids containing *nif* deletions indicated that the strains did not contain a second mutation. The products of *nifA* and *nifH* are believed to be involved in regulation (30), and the complementation pattern of these mutations may reflect an interaction of these two proteins.

All point mutations in *nifQ*, *nifS*, and *nifV* were very leaky except *nifS4618* and *nifV4940*. These mutations accounted for 21 of the 44 leaky point mutations. The other 23 were distributed roughly equally among the other *nif* genes. The two Mu-induced mutations in *nifQ* and the two left-most Mu insertions in *nifB*, *nifB5026* and *nifB5030*, also were leaky. Mu-induced mutations in *nifS* and *nifV*, the amber mutation *nifS4618* (see Table 2), and the ICR-induced mutation *nifV4940* were not leaky because they are polar onto *nifM*, the promoter-distal gene of this transcript. The chromosomal deletion Δ(his-nif)4303 also is a very leaky mutation, and it failed to complement *nifQ* point mutations but it did recombine with them. Figure 1 shows only six mutations in *nifQ* because leaky mutations were characterized from only the later selections for Nif⁻ strains.

Identification of *nifL*. In addition to the assignment of mutations to 13 complementation groups, we subdivided the *nifA* complementation group into two genes: *nifA* and *nifL*. This is based on several lines of evidence which suggest that all mutations we have isolated in *nifL* are polar onto *nifA*.

Except for *nifL2265* (12, 22), all 20 mutations in the right-most five deletion intervals of the *nifA* complementation group (labeled *nifL* on Fig. 1) are known to be polar—two amber mutations and 18 Mu insertions. Only about a third (12/33) of the mutations in the left two deletion intervals of the *nifA* complementation group (labeled *nifA* on Fig. 1) are known to be polar. Sixteen of the 18 strains with Mu insertions in *nifL* reverted to Nif⁺ at frequencies between

TABLE 2. *Suppressible and polar mutations*[a]

Mutation	Suppressible by:		Complementation pattern
	supF	*supD*	
nifQ5406	+[b]	+	*nifQ*
nifB4814	+	+	*nifQ nifB*
nifA4273	+	+	*nifA*
nifA4403	+	+	*nifA*
nifL4373	+	+	*nifA*
nifL4602	+	+	*nifA*
nifF4692	−	+	*nifF*
nifF4734	−	+	*nifF*
nifV5499	−	+	*nifM nifV*
nifS4618	+	+	*nifM nifV nifS*
nifN4740	+	+	*nifN*
nifN4753	+	+	*nifN*
nifE4096	+	+	*nifE*
nifE4669	+	+	*nifE*
nifE4701	+	+	*nifE*
nifK4717	+	−	*nifK*
nifD4396	+	+	*nifK nifD*
nifD4723	+	+	*nifK nifD*
nifJ4069	+	+	*nifJ*
nifJ4088	+	+	*nifJ*
nifJ4615	+	+	*nifJ*
nifJ4616	+	−	*nifJ*
nifJ4617	+	−	*nifJ*
nifJ4710	−	+	*nifJ*
nifB4780	−	−	*nifQ nifB*
nifV4940	−	−	*nifM nifV*
nifL2265	−	−	*nifA*
nifD4076	−	−	*nifK nifD*
nifD4709	−	−	*nifK nifD*

[a] Polarity of Mu-induced mutations is discussed in the text.

[b] +, Growth of the strain containing the *nif* mutation and plasmid with suppressor on N-free medium.

10^{-6} and 10^{-8}. In *E. coli*, strains with Mu insertions do not revert (37) except when the original mutation is suppressed by a mutation in another gene (7). In *K. pneumoniae*, no Nif$^+$ revertants were observed when 10^9 cells of 75 strains with Mu insertions in the other 12 *nif* complementation groups and five strains with Mu insertions in *nifA* were plated on N-free medium. Furthermore, Nif$^+$ revertants were isolated among survivors of heat inductions of strains with Mu insertions in *nifL*, but not in *nifA*.

The data suggest that a Nif$^+$ phenotype can be restored to strains with *nifL* mutations by relieving polarity of the *nifL* mutation. In the case of Mu insertions, this may ocur by deletion of the Mu and some *nifL* DNA such that *nifA* is again controlled by the *nifL nifA* promoter. A Nif$^+$ phenotype also may be restored by creation of a reinitiation site for RNA polymerase between *nifA* and the polar mutation in *nifL*. Both of these types of revertants still retain a *nifL* mutation, and therefore *nifL* is not required for growth on N_2. Some mutations in the right-most deletion interval of *nifA* may be polar *nifL* mutations. Alternatively, the *nifA nifL* region may be a single gene. This explanation requires a protein product with two regions, one essential to *nif* and one nonessential. Further characterization of these mutations awaits identification of a phenotype for nonpolar *nifL* mutations.

Polarity. When a mutation is polar, it interrupts transcription so that it fails to complement mutations in promoter-distal genes. We found that Mu-induced mutations in several cistrons exhibited polarity. Mu-induced mutations in *nifB* failed to complement mutations in *nifB* and *nifQ*. Mu-induced mutations in *nifV* did not complement mutations in *nifV* or *nifM*, whereas Mu insertions in *nifS* failed to complement *nifS*, *nifV*, and *nifM* mutations. Mu insertions in *nifE* failed to complement *nifN* and *nifE* mutations. Also, Mu insertions in *nifD* failed to complement *nifD* and *nifK* mutations, whereas Mu insertions in *nifH* failed to complement *nifH*, *nifD*, and *nifK* mutations. As described above, Mu insertions in *nifL* failed to complement *nifA*. Mu insertions in *nifQ*, *nifA*, *nifF*, *nifM*, *nifN*, *nifK*, and *nifJ* did not exhibit polarity onto additional genes in complementation tests.

Mu-induced mutations in *nifK* and *nifD* have a complex complementation phenotype. Mu insertions in *nifD* failed to complement mutations in *nifH* as well as *nifD* and *nifK* when the heterozygote was assayed for growth on N-free medium. However, Mu-induced mutations in *nifD* did complement *nifH* mutations but not *nifK* or *nifD* mutations if the heterozygote was assayed for acetylene reduction in vivo. This indicates that Mu-induced mutations in *nifD* are polar onto *nifK* but not onto *nifH*. Mu-induced mutations in *nifH* failed to complement *nifH*, *nifD*, and *nifK* mutations whether Nif was assayed by growth on N-free medium or by acetylene reduction. Mu induced mutations in *nifK* often showed weak complementation with point mutations in *nifD* and *nifH* when Nif was assayed by growth on N-free medium, but complemented mutations in *nifD* and *nifH* well when acetylene reduction was assayed.

All *nif* mutations were tested for suppressibility by two amber suppressors: SuA1P2, a double mutant of the Su3 (*supF*) gene on ϕ80*sus2* p*sup3* (15) and Su1 (*supD*). Derivatives of the plasmid RP4 carrying each of these suppressors were mated from *E. coli* strain N100 into all Nif$^-$ mutants, and the exconjugants were tested for their ability to grow on N-free medium. Twenty-four suppressible mutations in 12 *nif* genes were identified and are described in Table 2. Seven of these amber mutations were sufficiently polar that they failed to complement other promoter-distal mutations of that transcript. In addition, five polar, nonsuppressible mutations are listed in Table 2. Mutations *nifB4780* and *nifV4940* presumably are frameshift mutations since they were induced by, and were revertible by, ICR191F. A mutation in *nifL*, *nifL2265* (21, 22) and two mutations in *nifD*, *nifD4076*, and *nifD*4709 were polar mutations not suppressed by *supF* or *supD*. These polar mutations confirmed all the polarity observed by Mu-induced mutations, except that the amber mutations in *nifE* did not display sufficient polarity to fail to complement *nifN* mutations. Also, polar mutations in *nifD* were polar onto *nifK* but not onto *nifH* as was observed with Mu insertions in *nifD* on N-free medium. No polar mutations other than Mu insertions in *nifH* were detected.

Complementation analysis was performed with the plasmid deletions shown in Fig. 1. As expected, deletion mutations failed to complement mutations in genes for which they were deleted in part or completely. Deletions did complement mutations in genes not covered by the deletion except in some cases where the gene was part of a polycistronic transcript which had been interrupted by the deletion. All deletions whose left end point was in *nifB* (*Δnif-4349*, *Δnif-4926*, *Δnif-5253*, *Δnif-4832*, *Δnif-4332*, *Δnif-4534*, and *Δnif-4553*) recombined with but failed to complement *nifQ* mutations. Deletions which ended in *nifL*, *Δnif-4890*, *Δnif-5000*, *Δnif-5001*, *Δnif-4975*, *Δnif-4331*, *Δnif-4529*, and *Δnif-4980* were NifA$^-$. Deletions with end points in the *nifM nifV nifS* transcript (*Δnif-4333*, *Δnif-4338*, *Δnif-4972*, and *Δnif-4842*) all complemented mu-

tations in *nifM*. The three deletions ending in *nifE* (Δ*nif-4541*, Δ*nif-4334*, and Δ*nif-4976*) were polar onto *nifN*. The one deletion which entered *nifD* from the right (Δ*nif-4977*) was polar onto *nifK*. The two deletions whose left end points were in *nifH* (Δ*nif-5318* and Δ*nif-5319*) failed to complement *nifD* and *nifK* mutations. All deletions described were polar except the four ending in the *nifM nifV nifS* transcript. The deletion event in these four deletions may have fused the promoter-distal gene to another transcript either within or outside of *nif*.

The complementation pattern of the Mu-induced mutations, ICR-induced frameshift mutations, amber mutations, deletion mutations, and other polar mutations indicate that there are five polycistronic *nif* operons and two monocistronic operons. Transcription is right to left in the *nifQ nifB*, *nifA nifL*, *nifM nifV nifS*, *nifN nifE*, and *nifK nifD nifH* operons. The direction of transcription of the *nifF* and *nifJ* operons cannot be determined with the data presented here.

Cotransduction of *nif* mutations with *hisD*. The frequency of cotransduction of several mutations in each of the *nif* genes with the *hisD4226* mutation was measured. Phage P1*kc h1* was grown on strains containing each of the mutations shown in Table 3 and used to transduce UN209 to His$^+$. Two hundred transductants from each cross were scored for cotransduction of Nif$^-$ (Table 3). In general, the frequency of cotransduction of mutations in each gene was consistent with the order of the genes as determined by deletion mapping. Values obtained for mutations in *nifV* were anomalously high. However, the cotransduction frequencies of these three *nifV* mutations in Table 3 with a different *hisD* allele, *hisD4209* of UN1516, were equal to the frequency obtained with a *nifM* mutation. Therefore, the unusually high frequencies obtained with *nifV* mutations appear specific to the *hisD4226* allele. Mapping of plasmid deletions and chromosomal deletions indicate that *nifV* is between *nifM* and *nifS*. Excluding the *nifV* results, the *nif* genes were 72 to 37% cotransducible with *hisD4226*. The two-factor crosses of Table 3 show that there was no significant gap between any two *nif* genes.

Nif$^-$ strains described previously. We used our system of deletion mapping and complementation analysis to characterize approximately 75 strains containing NG-induced *nif* mutations isolated by St. John (Ph.D. thesis). In several of these strains, we were able to detect two *nif*-specific mutations by complementation analysis and by deletion mapping, demonstrating that NG causes closely linked double mutations as shown previously (16). UN316 and UN328 (33) were each found to contain two *nif* mutations. UN316 contains mutations in *nifD* and in *nifJ* and UN328 contains mutations in *nifB* and in *nifJ*. These mutations do not appear on Fig. 1. The mutations in strains UN142, UN150, UN318, UN364, and UN587 (*nif-4083*, *nif-4106*, *nif-4026*, *nif-4116*, and *nif-4066*) were characterized and found to map in the order previously described. Mutations in four strains described by Dixon et al. (12) and Kennedy (22) were analyzed. Mutations *nifA2263* and *nifE2296* mapped in *nifA* and *nifE*, respectively,

TABLE 3. *Cotransduction of nif mutations with hisD*[a]

nif gene	Allele	% Cotransduction with *hisD4226*	Mean %
nifQ	*4969*	74	72
	4970	69	
nifB	*4398*	61	
	4691	68	65
	4743	66	
nifA	*4381*	75	
	4683	63	66
	4739	59	
nifF	*4718*	54	
	4737	62	58
	4755	58	
nifM	*4690*	58	
	4750	52	53
	4820	49	
nifV	*4092*	84	
	4459	80	82
	4945	83	
nifS	*4282*	53	
	4389	55	53
	4618	51	
nifN	*4687*	48	
	4724	48	45
	4740	40	
nifE	*4629*	44	
	4675	45	43
	4712	39	
nifK	*4711*	44	
	4729	37	42
	4759	45	
nifD	*4685*	32	
	4697	35	37
	4762	45	
nifH	*4677*	42	40
	4714	38	
nifJ	*4727*	35	
	4747	41	39
	4823	40	

[a] P1*kc h1* was grown on strains containing each of the *nif* alleles listed and used to transduce UN209 (*hisD4226*) to His$^+$. Two hundred His$^+$ transductants were tested for cotransduction of the *nif* mutation by assaying growth on N-free medium.

and failed to complement *nifA* and *nifE* mutations, respectively. Like all other *nifL* mutations, *nifL2265* had a NifA$^-$ phenotype and mapped to the right of *nifA* mutations. In our complementation tests we saw no evidence of *nifL2265* being a partially transdominant *nif* mutation. The mutation *nifJ2319* was tentatively assigned to *nifJ* based on three factor crosses (22). Previous complementation analysis showed that it failed to complement mutations in *nifA*, *nifD*, and one mutation in *nifE* (12). We found that *nifJ2319* failed to complement only *nifJ* mutations and mapped in the middle deletion group in *nifJ*.

The chromosomal deletions Δ(*his-nif*)*4301*, Δ(*his-nif*)4302, Δ(*his-nif*)4303, Δ(*his-nif*)4304, and Δ(*his-nif*)*4305* were previously mapped by transduction and described as UN901, UN902, UN903, UN904, and UN906, respectively (4). We mapped these deletions in greater detail (Fig. 2) and found that the deletions of UN901, UN902, and UN904 did not delete *nifK4026* as previously described (4). UN903 containing Δ(*his-nif*)*4303*, which was thought to be Nif$^+$ (4), was further characterized, and although it is a very leaky Nif$^-$ strain, it failed to complement *nifQ* mutations. The mutation Δ(*his-nif*)*4648* is a P2 eductant (34) which was found to end in *nifV*.

DISCUSSION

We have isolated 390 independent Nif$^-$ mutants, excluding strains with deletion mutations, induced by a variety of mutagens. One hundred and thirty mutants contain Mu insertions, and the remainder contain point mutations. Complementation analysis was performed by crossing strains with plasmids containing *nif* mutations with Nif$^-$ Rec$^-$ strains. This analysis indicates there are 13 complementation groups and an additional gene, *nifL*, which are organized in seven operons. The identification of 24 amber mutations in 12 *nif* genes (Table 2) indicates there is a protein coded by each of these genes.

Forty-four Mu-induced deletions were isolated in the plasmid pTM4010 and were used to map the 390 chromosomal mutations (Fig. 1). Sixty-nine plasmids bearing point mutations and Mu insertions in *nif* were used to map the 99 chromosomal deletions in the *his-nif* region (Fig. 2). The two maps are in agreement and establish the order of *nif* genes as *his*... *nifQBALFMVSNEKDHJ*. The agreement of these two maps indicate that no rearrangement occurred in the *nif* region during the multistep construction of the parental *his-nif* plasmid, pRD1 (11).

This work confirms the order of four *nif* genes (excluding *nifG*) identified by St. John et al. (33) and five more described by Dixon et al. (12) and Kennedy (22). In addition, five new *nif* genes, *nifQ*, *nifM*, *nifV*, *nifS*, and *nifN*, have been identified. Four of these genes, *nifM*, *nifV*, *nifS*, and *nifN*, are in the "silent region" previously described between *nifF* and *nifE* (22). This silent region probably resulted from mapping only 22 mutations. Furthermore, our data for the cotransduction of *nif* mutations with a *hisD* mutation indicate that no large gaps occur between any two *nif* genes. This agrees with observations of Hsueh et al. (20).

Unlike Streicher et al. (36), we find no *nif*-specific mutation unlinked to *his*. We isolated eight Nif$^-$ strains in which the mutations were not linked to *his*, but all these strains were unable to use several organic N sources.

All strains with nonpolar point mutations in *nifQ*, *nifV*, and *nifS* are very leaky. Even *nifQ* mutations induced by Mu, which is known to inactivate the gene into which it inserts (37), are leaky. Two Mu-induced mutations in *nifB* also are leaky. They map at the *nifQ* proximal end of *nifB* and presumably at the very end of the *nifB* gene such that the product is produced in a partially functional form. Since they are polar on *nifM*, Mu-induced mutations in *nifV* and *nifS* are not leaky. This indicates that *nifQ*, *nifV*, and *nifS* are not totally essential to Nif expression but that they are required to achieve wild-type levels of N_2 fixation. The absence of nonpolar mutations in *nifL* suggests this gene is not required for the Nif$^+$ phenotype. If *nifL* codes for a *nif*-specific repressor (3), a mutation in *nifL* would not be expected to produce a Nif$^-$ phenotype. If *nifL* is not a gene for a repressor, nonpolar *nifL* mutations could be so leaky that none were isolated in our screen for Nif$^-$ strains. Proof of the existence of *nifL* awaits determination of gene function and determination of a phenotype for nonpolar mutations.

Previously *nifL* and *nifJ* were each defined by only one mutation, each of which had an unusual or complex complementation pattern (12). In addition, only two mutations in *nifF* had been described (12, 33). We have isolated many mutants with mutations in these genes (Fig. 1). All of our strains with mutations in *nifJ* fail to complement only *nifJ* mutations. Several mutations isolated by Dixon et al. were assigned to *nifD* (12) because they failed to complement UN316. However, our mapping experiments showed that UN316 contains two mutations, one in *nifD* and another in *nifJ*. It is possible that 7 of the 13 *nifD* mutations described by Dixon et al. (*nif-2025*, *nif-2031*, *nif-2032*, *nif-2035*, *nif-2053*, *nif-2102*, and *nif-2109*) may be *nifJ* mutations. We did not see any evidence of a *trans*-dominant phenotype (12) for *nifL* mutations. It

is possible that we might not detect a 10 to 50% reduction in nitrogenase activity in our plate assay for complementation.

An additional *nif* gene, *nifG*, which was defined by a single mutation, has been previously described (33). The strain with this mutation is unable to use a variety of N sources and is not cured by a Nif$^+$ plasmid, and the linkage of the mutation to *hisD* is doubtful (22). The mutation in this strain may not be *nif* specific.

Genes *nifB*, *nifA*, *nifF*, *nifM*, *nifN*, *nifE*, *nifK*, *nifD*, *nifH*, and *nifJ* are essential genes since mutations in any of them lead to an inability to grow on N_2. The existence of additional essential *nif* genes is doubtful since we have analyzed 346 nonleaky Nif$^-$ strains and found that all mutations can be assigned to the genes listed above. Because we have analyzed only 44 leaky mutations, it is possible that *nif* genes in which mutations produce a very leaky Nif$^-$ phenotype remain to be identified.

The polarity of suppressible mutations, ICR-induced mutations, Mu-induced mutations, and some deletions indicate there are seven operons, five of which are polycistronic: *nifQ nifB*, *nifA nifL*, *nifM nifV nifS*, *nifN nifE*, and *nifK nifD nifH*. Transcription is from right to left in all of these. *nifF* and *nifJ* are transcribed independently. C. Elmerich (personal communication) also has found that there is an operon of *nifK nifD nifH* which is transcribed from right to left based on results of the polarity of Mu-induced mutations.

Complementation analysis of mutations in the *nifK nifD nifH* operon is complex. Mu-induced mutations in *nifD* do not complement mutations in *nifK*, *nifD*, or *nifH*, and insertions in *nifK* complement mutations in *nifD* and *nifH* poorly when growth on N_2 is assayed. This may be due to regulatory roles of these proteins, subunit interactions of components I and II, or an improper ratio of these protein products in the cell.

We have not seen any significant intracistronic complementation in any of the complementation groups. It may be possible to detect this by constructing more derivatives of pTM4010 carrying mutations in each *nif* gene so that more mutations in the same gene can be tested.

Unlike Dixon et al. (12), we do not find that a large fraction of our mutations have complex complementation patterns. Differences in complementation patterns may be due, in part, to the different way in which their complementation tests were performed. For their experiments, plasmids containing *nif* mutations were mated into Rec$^+$ strains containing *nif* mutations in the chromosome (12). The heterozygotes were purified and tested for their ability to reduce acetylene. With two exceptions (*nifA4395* and *nifA4682*), all mutations characterized in this report which fail to complement more than one group are polar mutations. We do find that plasmids with *nifH* mutations, but not chromosomal *nifH* mutations, complement poorly. This may be due to a gene dosage effect. If so, the excess of defective *nifH* product, component II (30), may inhibit N_2 fixation.

In this work we have used two genetic techniques which may be applicable to other bacterial species, as well as *K. pneumoniae* and *E. coli*. We exploited the close linkage of a Tn*10* (Tet^r) insertion with *recA56* in a strain of *E. coli* isolated by L. Csonka to transduce all of our mutants to Rec$^-$. The *E. coli recA56* mutation reduced recombination in *K. pneumoniae* by 5×10^{-5} and might reduce recombination in other species as well. We also have used RP4 derivatives with amber suppressors isolated by J. Schell to rapidly screen all of our mutants for amber mutations. Because RP4 has a broad host range (10), this technique could be applied to many species of bacteria.

Strains containing mutations characterized in this paper have been used in the accompanying paper (30) to investigate the function of the *nif* genes. Strains with Mu insertions are especially useful since the insertion totally inactivates the product of the gene into which it has inserted (37). Strains containing derivatives of pTM4010 with Mu insertions also are being used to isolate *nif-lac* fusions by the technique of Casadaban (8) and λ*nif* specialized transducing phages. In addition, the deletions described here are useful for physical mapping of the *nif* genes.

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ADDENDUM

We have isolated 383 additional Nif mutants including deletion, Mu-induced, and point mutations in *nif*. These mutations confirm the mapping results and complementation analyses described here.

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