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Bacteriophage Mu-Induced Deletions in a Plasmid Containing the *nif* (N_2 Fixation) Genes of *Klebsiella pneumoniae*

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Five plasmids with insertions of a heat-inducible Mu prophage in a Mu-sensitive and P1-sensitive derivative of plasmid pRD1, a recombinant R factor containing the *his-nif* region of *Klebsiella pneumoniae*, were isolated and characterized. In one plasmid containing the Mu prophage integrated at the *his*-distal end of *nif*, selection for heat resistance resulted in the generation of deletions extending from the Mu prophage into the *nif* region. Thirty of these deletions were used to map 26 point mutations in *nif*.

The genetics of N_2 fixation has been studied extensively in *Klebsiella pneumoniae*. Point mutations (14, 18, 21) and Mu-induced mutations (15) in *nif* have been isolated and characterized. Mapping of *nif* mutations has been accomplished primarily by P1 transduction by measuring the frequency of cotransduction with *his* (14, 21, 22) and by ordering mutations by three-factor reciprocal crosses using *his* as the third marker (14, 18).

Deletion mapping permits a more defined and more efficient analysis of a genetic region than mapping by three-factor crosses. One method for isolating strains containing deletion mutations takes advantage of the properties of bacteriophage Mu, which has the unusual ability to integrate randomly in the bacterial chromosome (5, 6, 13, 23). Strains are isolated in which a heat-inducible Mu prophage is inserted within or near the genes of interest. Such lysogens die when grown at high temperature due to induction of phage development. Some of the heat-resistant survivors of induction have deletions which remove at least the immunity region of Mu and which often delete nearby bacterial DNA as well. In this way Mu has been used to isolate deletion mutations in *Escherichia coli* and in the *his-nif* region of *K. pneumoniae* (3, 12).

To permit rapid and simple mapping of *nif* point mutations in the *K. pneumoniae* chromosome, it would be useful to have a collection of deletion mutations in the *nif* region of a transferable plasmid. The plasmid RP41, redesignated pRD1 (7), is the ideal parent plasmid for the isolation of such deletions. pRD1 is a P-type plasmid constructed by recombination between an F'*his nif* and RP4 and can be transferred at high frequency to a variety of gram-negative bacteria (8). The plasmid pRD1 carries resistance to carbenicillin, tetracycline, and kanamycin and contains the *his-nif* region from *K. pneumoniae*. It also carries the markers *gnd, shiA*, and probably *rfb*, although it is not certain whether these genes come from *K. pneumoniae* or *E. coli* (8).

In this paper we describe the isolation and characterization of a set of deletion mutations in the *nif* region in a P1-sensitive derivative of pRD1, which were isolated by selecting for heat-resistant derivatives of a pRD1 plasmid containing a heat-inducible Mu prophage.

(Part of this work has been presented previously [M. Bachhuber, T. Malavich, and M. Howe, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, H77, p. 108].)

MATERIALS AND METHODS

Media. Recipes for LB and SB media (12), LC medium, P1 diluent, and B diluent (3) have been described. Soft agar contains twice-concentrated LC medium and 0.65% agar. Minimal medium, described previously (18, 25), was modified to contain 13.9 g of Na_2HPO_4, 1.7 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4.8 mg of $FeCl_3$, 0.25 mg of $NaMO_4$, and 2 g of NaCl per liter. For *K. pneumoniae*, 0.6% sucrose was used as the carbon source; for *E. coli*, 0.4% glucose was used, and the medium was supplemented with 1 μg of thiamine per ml. Fixed N was present as 0.2% ammonium acetate when needed. Amino acids were added to 20 μg/ml when required. All solid media contained 15 g of agar (Difco) per liter, except N-free media, which contained 13 g of purified agar (Difco) per liter.

When necessary, filter-sterilized antibiotics were added to the medium to the following concentrations: carbenicillin (Pfizer, New York, N.Y.) at 200 μg/ml, tetracycline (Lederle, Pearl River, N.Y.) at 10 μg/ml, spectinomycin (a gift of G. B. Whitfield, Upjohn Co., Kalamazoo, Mich.) at 200 μg/ml, kanamycin at 25 μg/ml, nalidixic acid at 40 μg/ml, and chloramphenicol

at 12.5 μg/ml. The latter three antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.

Bacteriophage. P1*kc* was obtained from W. H. McClain. P1CM*clr*100 (17) and Mu *cts Cam*4005, Mu *cts Dam*3801, Mu c^+ *Mam*1124, and Mu *c*25 have been described (1, 12).

Bacterial strains. *K. pneumoniae* M5a1 was obtained from P. W. Wilson. All Nif⁻ mutant strains were isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (18). The strains of *K. pneumoniae* and *E. coli* K-12 used in this work are listed in Table 1. Mutations *nif-4026* through *nif-4229* shown in Tables 2 and 3 were isolated by R. T. St. John (Ph.D. thesis, University of Wisconsin, Madison, 1973). The map order and phenotypes of mutations *nif-4026, nif-4066, nif-4083, nif-4106,* and *nif-4116* have been described previously (18).

Isolation of Mu lysogens. Phage lysates were spotted on cells suspended in soft agar over LC plates. After overnight incubation at 32°C, cells were purified from the area of the lawn spotted with phage. Lysogens were identified by their immunity to infection by

TABLE 1. *Bacterial strains*

Strain	Genotype/phenotype	Source or reference
K. pneumoniae M5a1		
UN209	*hisD4226*	
UN562	*pro* Δ(*rfb-gnd-his-nif*)	Δ16 of ref. 20
UN2161	UN562(pTM4039)	
UN2162	UN562(pTM4040)	
UN2163	UN562(pTM4086)	
UN2164	UN562(pTM4087)	
UN2165	UN562(pTM4088)	
UN2166	UN562(pTM4089)	
UN2167	UN562(pTM4090)	
UN2168	UN562(pTM4091)	
UN2169	UN562(pTM4092)	
UN2170	UN562(pTM4093)	
E. coli K-12		
CSH54	Δ(*pro-lac*) *trp pyrF his rpsL supF*	Cold Spring Harbor Laboratory Collection
JC5466(pRD1)	*trp his recA56 rpsE* (Carb^r Tet^r Kan^r *gnd his nif shiA*)	R. Dixon (8)
M107	*lac supD rpsL*	12
MH1674	Δ(*pro-lac*) *trp sup*$^+$ *nalA* (Mu c^+ *Sam*6002)	BU8848 of ref. 16
UQ26	*his ara galK malA xyl mtl rpsL* (λ⁻) *sup*$^+$	SB1801 of ref. 10
UQ27	*his ara galK malA xyl mtl rpsL* (λ⁻) *sup*$^+$ *nalA*	Spontaneous Nal^r of UQ26
UQ28	*his ara galK malA xyl mtl rpsL* (λ⁻) *sup*$^+$ *rpsE*	Spontaneous Spc^r of UQ26
UQ29	UQ27(pTM4010)	
UQ30	UQ27(pTM4012)	
UQ31	UQ27(pTM4013)	
UQ32	*his ara galK malA xyl mtl rpsL* (λ⁻) *sup*$^+$ *rpsE* (Mu c^+ *Mam*1124)	
UQ33	UQ27(pRD1)	
UQ34	UQ28(pTM4010)	
UQ35	*his ara galK malA xyl mtl rpsL* (λ⁻) *sup*$^+$ *nalA* (Mu *cts Cam*4005) (pTM4010)	
UQ36	*his ara galK malA xyl mtl rpsL* (λ⁻) *sup*$^+$ *nalA* (Mu *cts Dam*3801) (pTM4010)	
UQ37	UQ32(pTM4011)	
UQ38	UQ32(pTM4012)	
UQ39	UQ32(pTM4013)	
UQ40	UQ32(pTM4014)	
UQ41	UQ32(pTM4015)	
UQ42	UQ27(pTM4011)	
UQ43	UQ27(pTM4014)	
UQ44	UQ27(pTM4015)	
UQ45–UQ50	UQ27 containing plasmids pTM4016–pTM4021	
UQ51	UQ27(pTM4023)	
UQ52–UQ62	UQ27 containing plasmids pTM4025–pTM4035	
WD5021	*gal lac sup*$^+$ *rpsL*	12

Mu in a cross-streak assay or by their ability to release plaque-forming phage (3, 12).

Isolation of plasmids containing a Mu prophage. A modification of the technique of Razzaki and Bukhari (16) was used to isolate plasmids with Mu insertions. A Mu *cts Cam*4005 lysogen (strain UQ35) and a Mu *cts Dam*3801 lysogen (strain UQ36) were used as donors of the plasmid. The donors and recipient strain (UQ32) were grown to approximately 2×10^8 to 5×10^8 cells per ml at 32 and 37°C, respectively. A 0.1-ml portion of the donor and 0.1 ml of the recipient were mixed together on an LC plate, and the mating mixture was incubated for 6 h at 42°C. Control matings under noninducing conditions (32°C) also were performed. The mating mixtures were washed with minimal medium by centrifugation and diluted in and plated on minimal medium appropriate for selection of His$^+$ Spc^r Carb^r exconjugants. After the plates were incubated for 2 days at 37°C, they were replicated to a lawn of strain M107 (Su$^+$) to detect phage released spontaneously from exconjugants as a result of complementation between a Mu *amC* or *amD* prophage on the plasmid and the chromosomal Mu *amM* prophage.

Prophage marker rescue tests. Marker rescue tests were performed by spotting lysates of Mu *am* phage from each complementation group (*A* through *S* alphabetically and *lys*) on lawns of heat-resistant, Mu-sensitive mutant strains derived from Mu *cts* lysogens and on Su$^-$ and Su$^+$ control strains (12).

Isolation of P1CM*clr*100 lysogens. P1CM*clr*100 lysogens were isolated and identified as described by Rosner (17) by virtue of the chloramphenicol resistance and heat sensitivity that the P1 confers on the lysogen.

Assays of Nif activity. Assays of Nif activity by acetylene reduction (4) and by growth on N-free medium (21) have been described. Carbenicillin, tetracycline, and kanamycin were added to the growth medium when testing the Nif activity of strains containing plasmids.

Mutagenesis. Mutageneses with UV light (19), nitrous acid (19), and mitomycin C (2) were performed as described previously. All incubations were at 32°C.

Isolation of Mu-induced deletions. (i) In liquid. Strains containing plasmids with Mu insertions were grown at 32°C in SB to 4×10^8 cells per ml. An equal volume of 60°C medium was added, and the cultures were shifted to 42°C for 7 h. Cells and cell debris were pelleted by centrifugation, resuspended, and diluted in and plated on minimal medium containing one antibiotic. Plates were incubated for 2 days at 42°C. The survivors were tested for Mu sensitivity by cross-streaking against Mu *c*25 and tested for phage release by replicating onto a lawn of strain M107.

(ii) On solid medium. Overnight broth cultures of strains containing plasmids with Mu insertions were diluted and plated on minimal medium with carbenicillin, tetracycline, and kanamycin. After incubation of the plates at 32°C for 2 days, single colonies were transferred to minimal medium containing one antibiotic. These plates were incubated at 32°C until patches were just visible (about 12 to 18 h). The plates were then replicated to similar plates which were incubated for 2 days at 42°C. A single heat-resistant colony was picked from each patch and purified at 42°C. Purified colonies were tested for phage release on strain M107 and for Mu sensitivity by cross-streaking against Mu *c*25.

(iii) By mating. Strains UQ43 and UN562 were grown to 2×10^8 to 5×10^8 cells per ml in LC. A 0.1-ml amount of each was mixed on an LC agar plate, which was incubated for 6 to 7 h at 34°C. The mated mixture was streaked on minimal medium with proline and kanamycin and incubated at 42°C for 2 days. Single colonies were tested for their Nif phenotype by assaying their ability to grow on N-free medium.

Matings. *E. coli* donors were grown to 2×10^8 to 3×10^8 cells per ml in LB at 37°C, or at 32°C if the strain contained a heat-inducible Mu prophage. Recipients were grown in LB, if *E. coli*, or in LC, if *K. pneumoniae*, to 6×10^8 cells per ml. A 0.1-ml portion of donor and 0.1 ml of recipient were mixed on an LC agar plate and incubated at 37 or 32°C for 5 to 6 h. Cells from the mating mixture and control unmated cells were then streaked onto selective medium.

The above technique was modified for matings of the deletion mutant strains with the many Nif$^-$ mutant strains. A 0.1-ml amount of an overnight culture of a recipient strain was spread over the surface of an LC plate. A 10-μl amount of each of 25 different donor strains was spotted on the lawn of recipient cells. These mating plates, together with unmated controls, were incubated overnight at 30°C and then replicated to N-free medium containing one antibiotic. The exconjugant was scored as Nif$^+$ if growth occurred in the mixed patch of donor and recipient on N-free medium but not in the unmated controls. A Nif$^-$ phenotype was scored if no growth occurred. Growth did occur when these plasmids were mated to His$^-$ Nif$^+$ recipients.

Transduction by P1*kc*. P1 lysates were grown on plates using the confluent lysis method (3), with a ratio of cells to phage of 10:1. Since the frequency of transduction with nonlysogenic recipients was very low, all recipients were made lysogenic for P1CM*clr*100. Transductions were done by the method of Wolf et al. (24), using a multiplicity of infection of 5.

RESULTS

Isolation of a mutant of pRD1 which no longer confers resistance to Mu and P1.

When plasmid pRD1 was transferred into a variety of strains of *E. coli* sensitive to Mu and P1, the strains lost the ability to grow Mu and P1. This might be due to expression of the *rfb* locus on the plasmid, resulting in an altered cell wall. For this reason we suspect that *rfb*, which is closely linked to *gnd*, *his*, and *nif* in *K. pneumoniae* (20), may be an additional marker in pRD1. To facilitate the use of Mu and P1, it was necessary to isolate an altered plasmid which did not confer resistance to these phages. Selection of P1-sensitive mutant strains of enteric bacteria has been described (11) and is based on the selection of kanamycin-resistant derivatives arising after infection of cultures with

P1KM*clr*100, which confers kanamycin resistance to its lysogens. This technique was modified for the isolation of P1-sensitive mutations in pRD1 by substituting P1CM*clr*100 for P1KM*clr*100 because the pRD1 plasmid itself confers resistance to kanamycin. An unmutagenized culture of strain UQ33 was infected with P1CM*clr*100, grown overnight at 32°C, and plated on minimal medium containing carbenicillin, tetracycline, kanamycin, and chloramphenicol. P1CM*clr*100 lysogens were isolated at a frequency of 10^{-8} and were found to be sensitive to Mu. When the plasmid was transferred to several strains of *E. coli*, all remained sensitive to Mu and P1. This confirmed the hypothesis that the mutation allowing phage sensitivity was located on the plasmid. This new P1-sensitive, Mu-sensitive plasmid was designated pTM4010.

Stability of the plasmid. The stability of pTM4010 was tested by mating a plasmid-containing strain to a recipient strain, selecting for transfer of only a small number of plasmid markers, and then scoring for cotransfer of unselected markers. When pTM4010 was transferred to strains of *E. coli* or *K. pneumoniae*, and exconjugants were selected for only the three plasmid-encoded antibiotic resistances, 15% of the 250 exconjugants tested were His^- and Nif^-. The remaining 85% were both His^+ and Nif^+. If, in addition to selecting resistance to the three antibiotics, either His^+ or Nif^+ was selected, the exconjugants always contained the unselected Nif^+ (250 tested) and His^+ (150 tested) markers. Therefore, it seems that the *K. pneumoniae his-nif* region can be lost from the plasmid either before or during conjugation. However, the isolation of such deleted plasmids can be prevented by selecting for either His^+ or Nif^+ antibiotic-resistant exconjugants. We did not see any segregation of markers from pRD1 when matings were performed with a Rec^- or Rec^+ donor, and we have not determined why pTM4010 is different from pRD1 in this regard.

Isolation of plasmids with Mu insertions. Razzaki and Bukhari (16) described a technique for easily isolating Mu insertions in transmissible plasmids. When a Mu *cts* lysogen is heat induced, the prophage undergoes vegetative growth. Approximately 10 copies of progeny Mu DNA become integrated at different sites in the chromosome (16). If a plasmid is present, it also becomes a target for Mu integration. Plasmids with Mu insertions can be recovered by mating the induced lysogen to an appropriate recipient and scoring the exconjugants for the presence of the prophage which was originally integrated in the chromosome of the donor strain.

This technique was used to obtain pTM4010 plasmids carrying a Mu prophage. A Mu *cts Cam*4005 lysogen (strain UQ35) and a Mu *cts Dam*3801 lysogen (strain UQ36) were used as donors of the plasmid. The recipient (strain UQ32) contained a Mu c^+ *Mam*1124 prophage to prevent zygotic induction in exconjugants receiving a plasmid containing a Mu *cts am* prophage. The particular *C* or *D* amber mutations were chosen because they were not leaky and they gave low rates of reversion and because lysogens of these amber mutant phages gave low frequencies of survivors after heat induction. The defective Mu prophages were used to prevent phage production in the subsequent heat selection.

The plasmid transferred His^+ and $Carb^r$ from both donors with a frequency of 10^{-1} per donor cell. Approximately 2,000 exconjugant colonies from each mating under inducing and noninducing conditions were tested for phage release arising due to complementation between the two defective Mu prophages. Five strains able to release phage were isolated from matings performed under inducing conditions, whereas none were isolated from matings done under noninducing conditions. Four of these (strains UQ37 through UQ40) were obtained from the mating with the Mu *cts Cam* donor, and they contained plasmids (pTM4011 through pTM4014) with insertions of the Mu *cts Cam* prophage. Plasmid pTM4011 was Nif^-. One strain (UQ41) was isolated from the mating with the Mu *cts Dam* donor. The plasmid in this strain (pTM4015) contained the Mu *cts Dam* prophage and was Nif^-. All five plasmids were His^+ and conferred resistance to carbenicillin, tetracycline, and kanamycin.

In addition to these five strains which behaved in the expected manner, an unusual class of exconjugants was also observed. This class, which made up 0.5% of the exconjugants, released phage when replicated to a lawn of indicator cells. However, the original colony never regrew on the selective plate used as the master for replication, nor could viable exconjugants be isolated from the location of the original colonies. What caused the death of these colonies is not known.

To obtain additional evidence that each of these five strains (UQ37 through UQ41) contained a plasmid with a M *cts am* insertion, the plasmids were transferred to three different types of strains, and exconjugants were tested for characteristics indicative of the presence of the prophage on the plasmid. When the plasmids were transferred to a His^- Su^- strain (UQ27), all 40 His^+ exconjugants from each mating died at 42°C and showed only a low level of phage release on an Su^+ strain (M107) and no phage

release on an Su⁻ strain (WD5021). This phage release was due to transfer of the plasmid into the Su⁺ cells with subsequent zygotic induction. These results are as expected if these plasmids contain a Mu *cts am* prophage. When the plasmids were transferred into an Su⁻ Mu c^+ *am* lysogen, strain MH1674, all of the exconjugants showed high-level phage release on Su⁺ cells (strain M107) but not on Su⁻ cells (strain WD5021), proving that the plasmid contained a prophage able to complement the amber prophage in the chromosome. When each of the five plasmids was transferred to a His⁻ Su⁺ strain (CSH54), the exconjugants died at high temperature and showed phage release on an Su⁺ lawn, but not on an Su⁻ lawn, as expected. We conclude, therefore, that plasmids pTM4011 through pTM4015 each contain a Mu *cts am* prophage.

Since some loss of *his* and *nif* had been observed for pTM4010, each of the five plasmids containing Mu was tested for maintenance of Mu in the plasmid. Strains UQ30, UQ31, and UQ42 through UQ44 were grown for 40 generations under nonselective conditions in LB and then were plated on LC plus carbenicillin. About 750 colonies from each strain were replicated to test for Tet^r, Kan^r, His⁺, Nif⁺, and phase release. All colonies possessed all of the plasmid markers including Mu; therefore, loss occurred at a frequency <0.13% in 40 generations.

Isolation of deletion mutations. Some heat-resistant mutant strains derived from Mu *cts* lysogens contain deletions of the prophage and neighboring bacterial genes (12). The strains no longer release phage and are sensitive to superinfection by Mu. In these strains, the deletion removes at least the *c* gene immunity end of the prophage and extends for variable lengths into adjacent bacterial genes. To obtain strains containing deletions extending into *nif*, we have isolated heat-resistant, Mu-sensitive mutant strains from strains containing plasmids with a Mu insertion.

Heat selections were performed in SB with each of the five strains containing a Mu *cts am* inserted into pTM4010 (UQ30, UQ31, UQ42, UQ43, and UQ44). One hundred and fifty heat-resistant survivors from each of the five strains were tested for Mu sensitivity. Thirty-four to 90% of the heat-resistant survivors were sensitive to Mu and did not release phage, suggesting that they contained deletions of the Mu prophage. Marker rescue tests were performed on eight Mu-sensitive mutants from each strain to determine which prophage genes were present. Deletions removed contiguous groups of markers beginning at the *c*-gene immunity end and extended for varying distances into the Mu prophage or even removed all Mu markers. Each of the heat-resistant, Mu-sensitive mutant strains was tested for its ability to grow on N-free medium. Strains isolated from strain UQ42 and UQ44 were Nif⁻ like their parental strains. Among 450 isolates from the other three strains, only two were Nif⁻. These arose from strain UQ43 and were designated UQ45 and UQ46.

Since many deletion mutant strains isolated in the above heat selections were likely to be siblings, further heat selections were done by the replica plating technique described in Materials and Methods. Approximately 350 heat-resistant, Mu-sensitive mutant strains from each of the three Nif⁺ strains (UQ30, UQ31, and UQ43) were tested for their Nif phenotype. Only 10 strains, designated UQ47 through UQ56, were Nif⁻, and all of these were derived from strain UQ43. All were still His⁺ and resistant to all three antibiotics.

The replica plate heat selection technique was modified in an effort to obtain Nif⁻ mutant strains from strains UQ30 and UQ31. If the orientation of markers on the plasmids of these strains were Mu ... *his* ... *nif*, plasmids that were His⁺ and had a deletion originating within Mu and extending into *nif* could not be isolated. Since all heat selections had been done demanding His⁺, it would not be possible to isolate Nif⁻ mutant strains if the above orientation of markers existed. Therefore, the replica plate heat selection was performed on LC plates containing carbenicillin. One hundred and fifty heat-resistant, Mu-sensitive mutants each were isolated from strains UQ30 and UQ31. Ten from strain UQ30 and 12 from strain UQ31 were Nif⁻ and His⁻. Crosses of strains containing these deletion mutations with strains containing *nif* point mutations yielded no Nif⁺ recombinants, suggesting that the deletions completely removed *nif*. Presumably they resulted from loss of a large section of the *K. pneumoniae* DNA as well as loss of Mu from the plasmids.

Since the frequency of Nif⁻ mutant strains from strain UQ43 was very low (about 3% of the heat-resistant, Mu-sensitive mutant strains), cultures of strain UQ43 were mutagenized with nitrous acid, mitomycin C, or UV light. Each of these mutagens is known to increase the frequency of deletion mutations (2, 19). Mutagenized cultures were spotted on LC plates. As soon as growth was detectable, plates were replicated to minimal medium with one antibiotic. These plates were then treated by the previously described method for the replica plate heat selection. Although mutageneses were effective in killing cells, they did not increase the frequency of Nif⁻ mutant strains among heat-resistant survivors. Two of 80 heat-resistant, Mu-sensitive

mutants arising after mitomycin C mutagenesis were Nif⁻, strains UQ57 and UQ58. Three of 80 from nitrous acid mutagenesis, strains UQ59, UQ60, and UQ61, were Nif⁻. One of 200 from UV mutagenesis was Nif⁻, strain UQ62.

Since the frequency of Nif⁻ strains among heat-resistant survivors was so low, a different procedure was used to try to simplify their isolation. It was based on an initial enrichment for deleted plasmids followed by heat selection and direct scoring of the heat-resistant survivors for their Nif phenotype instead of their Mu sensitivity. To facilitate the scoring of Nif, the latter steps were performed in a *K. pneumoniae* host, strain UN562. The enrichment procedure involved the transfer of the Mu-containing plasmid from strain UQ43 to strain UN562 by conjugation at 34°C. The rationale for this procedure was that at 34°C zygotic induction of the transferred Mu *cts* prophage should be extensive and result in killing of the majority of the exconjugants. Plasmids deleted for the immunity end of Mu would be unable to kill upon transfer and therefore would be enriched in the surviving exconjugant population. The subsequent heat selection would eliminate those strains with plasmids which were able to survive zygotic induction and, after replication to N-free medium, would allow the detection of strains containing Nif⁻ plasmids. Although the hypothesis was that the deleted plasmids would be present in the donor population before mating, it is likely that some deletions were generated as a result of the zygotic induction itself. Regardless of the mechanism of generation of the deletions, the procedure itself was successful, resulting in a frequency of Nif⁻ plasmids of almost 10%. Strains UN2161 through UN2170 were obtained using this selection procedure. The Nif⁻ plasmids from these strains were transferred into *E. coli* strain UQ27, and Mu marker rescue tests were performed to determine which prophage markers were deleted. In all strains all Mu markers were missing.

Mapping of Mu-induced deletions extending into *nif*. The extent of each deletion into the *nif* region of each mutated plasmid was determined by crossing each deletion with various *nif* point mutations using conjugation and transduction. All plasmids were transferred to *K. pneumoniae* strain UN562 so that in subsequent mating and transduction experiments with *K. pneumoniae* recipients there would not be any problem with restriction. Matings were done on LC plates, which were then replicated to N-free medium to detect Nif⁺ recombinants. The mapping results are shown in Table 2. The data are arranged to reflect the probable map

TABLE 2. *Mapping of Mu-induced deletions into nif*[a]

Plasmid no.	*nif* point mutations								
	4064 4106	*4176 4227*	*4031 4066 4070 4078 4105*	*4027 4093 4229*	*4086 4172 4047*	*4195 4197*	*4026 4115*	*4060 4083 4108 4111 4116*	*4057 4219*
Nif⁺ control									
pTM4010	+	+	+	+	+	+	+	+	+
Original Mu insertions									
pTM4011	+	+	−	−	−	−	−	−	−
pTM4012	+	+	+	+	+	+	+	+	+
pTM4013	+	+	+	+	+	+	+	+	+
pTM4014	+	+	+	+	+	+	+	+	+
pTM4015	−	−	−	−	+	+	+	+	+
Deletions derived from pTM4014									
pTM4040	+	−	−	−	−	−	−	−	−
pTM4023, pTM4087, pTM4092	+	+	−	−	−	−	−	−	−
pTM4091	+	+	+	−	−	−	−	−	−
pTM4016, pTM4021, pTM4039	+	+	+	+	−	−	−	−	−
pTM4017, pTM4088	+	+	+	+	+	−	−	−	−
pTM4031, pTM4034, pTM4093	+	+	+	+	+	+	−	−	−
pTM4089	+	+	+	+	+	+	+	−	−
pTM4020, pTM4035, pTM4086, pTM4090	+	+	+	+	+	+	+	+	−
pTM4018, pTM4019, pTM4025–pTM4030, pTM4032, pTM4033	−	−	−	−	−	−	−	−	−

[a] Each plasmid, in a background of strain UN562, was transferred to strains containing the *nif* point mutations shown. A positive result indicates growth of the merodiploid on N-free medium.

order of the nine clusters of mutations. A positive result indicates that at least recombination and possibly complementation occurred to restore the Nif$^+$ phenotype. All plasmids were transferred to UN209, a His$^-$ Nif$^+$ strain. All exconjugants were His$^+$ Nif$^+$, indicating that each plasmid transferred well upon mating and no plasmid had a dominant *nif* mutation.

Nine of the plasmids listed in Table 2 are totally deleted for *nif.* Sixteen additional total *nif* deletions were obtained from heat-resistant exconjugants in the mating of strain UQ43 to strain UN562. These 16 strains were not examined further. The 25 plasmids totally deleted for *nif* represent 58% of all Nif$^-$ plasmids isolated.

Several plasmids were also mapped by transduction against the *nif* point mutations *4106, 4066, 4026, 4083,* and *4116,* whose order was previously determined by three-factor crosses (21). P1 was grown on *K. pneumoniae* strains containing the plasmids pTM4010 through pTM4017, pTM4020, pTM4021, pTM4023, and pTM4025 through pTM4037. The phage was used to transduce the five Nif$^-$ strains to Nif$^+$. The results obtained by transduction (Table 3) agreed with results obtained by conjugation and confirmed the previous map order.

Heat-resistant, Mu-sensitive mutant strains were isolated from UQ42 and UQ44, the two strains which carried plasmids that, upon insertion of Mu, were Nif$^-$. Sixty mutants from each strain were mapped by the mutations listed in Table 2 to determine if any had extended deletions into *nif.* All mutant strains isolated from strain UQ42 had deletions which mapped like the deletion of the parental plasmid. All but two mutant strains from UQ44 had deletions which mapped like the deletion of the parental plasmid. The other two strains had total deletions of *nif.*

DISCUSSION

A P1-sensitive, Mu-sensitive derivative of plasmid pRD1, pTM4010, was isolated. This derivative should enhance the usefulness of the plasmid not only by allowing the use of bacteriophage Mu for genetic manipulations, but also by enabling the use of P1 transduction in strains containing the plasmid. This latter capability should be particularly useful for merodiploid analysis of *nif* functions, since it will greatly simplify the transfer of existing defined *nif* point mutations and deletion mutations from the chromosome into the plasmid.

Derivatives of pTM4010 containing Mu insertions were isolated using the plasmid transfer system first described for F′*pro lac* by Razzaki and Bukhari (16). In the case of F′*pro lac,* the frequency of transferred plasmids containing Mu was 1 to 5%, whereas the frequency for pTM4010 was only 0.6%. Eighty-five percent of the exconjugants containing pTM4010 and Mu were unstable with regard to cell viability on the original selective medium, and only five exconjugants containing Mu stably integrated into the plas-

TABLE 3. *Mapping of Mu-induced deletions in nif by P1 transduction*[a]

Plasmid no.	*nif* point mutation				
	4106	*4066*	*4026*	*4083*	*4116*
Nif$^+$ control					
pTM 4010	180	320	50	620	410
Original Mu insertions					
pTM4011	63	0	0	0	0
pTM4012	50	205	110	205	125
pTM4013	510	360	105	320	290
pTM4014	235	550	50	267	216
pTM4015	0	0	270	900	>1,000
Deletions derived from pTM4014					
pTM4023	175	0	0	0	0
pTM4016	75	44	0	0	0
pTM4021	350	150	0	0	0
pTM4017	415	270	0	0	0
pTM4031	750	770	0	0	0
pTM4034	550	334	0	0	0
pTM4020	410	750	30	110	75
pTM4035	730	815	87	114	102
pTM4018, pTM4019, pTM4025–pTM4030, pTM4032, pTM4033	0	0	0	0	0

[a] Phage P1 grown on UN562 containing each of the plasmids was used to transduce five strains with each of the *nif* point mutations to Nif$^+$. Numbers presented are the number of Nif$^+$ transductants arising per 2×10^8 plaque-forming units of infecting P1.

mid were recovered. Figurski et al. (9), who used a similar technique to isolate Mu insertions in the plasmid RK2, also observed instability of Mu-containing plasmids. Seventy to 80% of their Mu-producing exconjugants segregated non-Mu-producing colonies at high frequency. The reason for this instability is not known, but it might be due to insertion of site-specific induction or deletion of the prophage.

Two of the Mu-containing plasmids derived from pTM4010, pTM4011 and pTM4015, were Nif⁻ when isolated and already contained partial deletions of *nif*. Presumably they arose by Mu-promoted deletion analogous to that observed in the *lacZ* region (13).

When heat selections were performed to isolate Mu-induced deletions in the remaining three plasmids, only one plasmid, pTM4014, gave deletions extending into *nif*. All deletions in this plasmid entered the *nif* region from the *his*-distal end of *nif*, suggesting that the order is *his* ... *nif* ... Mu. The inability to isolate Nif⁻ deletions in plasmids pTM4012 and pTM4013 might be due to the location of the Mu prophage. If genes essential for plasmid maintenance were located between *nif* and the Mu prophage, it would not be possible to isolate plasmids containing deletions extending from Mu into *nif*.

The extent of each deletion into *nif* was determined by assaying for the production of Nif⁺ recombinants in conjugational crosses of each of 30 plasmid-containing strains with 26 strains carrying *nif* point mutations. The results were confirmed in crosses done by P1 transduction for selected plasmids and point mutations. The results allowed the point mutations to be separated into nine deletion groups, which confirmed and extended the mapping results described previously (3, 18).

Several of the deletion end points were not distinguishable from each other with the small number of point mutations used. It is likely that new distinctive end points will be discovered among these when they are mapped using a greater number of point mutations. The existence of such deletions on the pTM4010 plasmid should provide a rapid and accurate way to map large numbers of *nif* point mutations.

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