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Mutational Alteration of a Nitrogen-Fixing Bacterium to Sensitivity to Infection by Bacteriophage Mu: Isolation of *nif* Mutations of *Klebsiella pneumoniae* M5a1 Induced by Mu

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The nitrogen-fixing bacterium *Klebsiella pneumoniae* M5a1 is not sensitive to infection by bacteriophage Mu. A mutant of *K. pneumoniae* that is sensitive to Mu infection was isolated. Several Mu-induced auxotrophic mutations of *K. pneumoniae* including *nif*, *trp*, and *rtl* were isolated and genetically characterized. Evidence is presented that the Mu-induced mutations of *nif* arise as the result of insertion of Mu within (or near) the *nif* operon(s). The *rtl* locus, which determines the ability to utilize ribitol as a carbon source, was found to be linked to *nif* loci.

Bacteriophage Mu-induced mutations have been used extensively to study the genetic organization of several operons in *Escherichia coli* (11). It would be of considerable interest to construct a series of Mu-induced *nif* mutations to study the *nif* operon(s) of *Klebsiella pneumoniae* M5a1. Unfortunately, none of the *Klebsiella* strains known to fix nitrogen was found to be sensitive to Mu infection (R. C. Valentine, personal communication; 16).

In this paper, the isolation of a *K. pneumoniae* M5a1 strain sensitive to Mu infection is described. Also, some strains with deletions of the histidine region were found to be partially sensitive to Mu. Isolation of Mu-induced *nif*, *trp*, and *rtl* mutations and their genetic analysis are presented in this paper.

MATERIALS AND METHODS

Strains. Bacterial strains used are listed in Table 1. Phage Mu-1 is referred to as Mu throughout this paper. λNN*pgal8h*^{424} was a generous gift from F. Ausubel.

Media. Most of the media used have already been described (15, 16, 19). M9 and E media were used as minimal media. Slightly modified Yoch and Pengra medium (19, 26) was used as the nitrogen-free medium (Y). The above media were solidified by adding agar (Difco) to a concentration of 1.5%. Nutrient broth and nutrient agar were used as maximal media. φ broth contained (per liter): 10 g of tryptone (Difco), 1 g of yeast extract, 5 g of sodium chloride, 2.5 g of magnesium sulfate, 0.75 g of calcium chloride, and 5 g of maltose. φ soft agar is φ broth containing 50 μg of thymine per ml and solidified with 6 g of agar (Difco) per liter. φ plates differed from φ broth in that maltose was not added and 10 g of agar (Difco) per liter were used.

Culture conditions. Culture conditions of bacteria and phages have already been described (15, 16). For assaying phage, fresh overnight cultures of bacteria grown in broth at 25°C without forced aeration were used.

Mutagenesis. Ethyl methane sulfonate and *N'*-methyl-*N*-nitro-nitrosoguanidine (NTG) were used as described previously (13, 18). Mu was used in the following manner to isolate mutations: a 0.1-ml sample of phage Mu lysate (~10^9/ml) was spotted on a lawn of a Mu-sensitive strain and the plate was incubated at 30°C overnight to allow phage growth. The lysed area was scooped out and suspended in about 5 ml of φ broth and incubated overnight at 30°C. When wild-type Mu was used, the cultures were incubated at 37°C instead of at 30°C. The phage-infected culture was used as the source of all Mu-induced mutations. Auxotrophic mutations were isolated with (18) or without penicillin enrichment. Nif$^-$ mutations were isolated essentially as described by Streicher et al. (21).

Mating conditions. Matings were done as described before (15, 16).

Isolation of strain BK383. The galactose transduction method of Streicher et al. (23) was employed in the isolation of a restriction-deficient strain BK383. One restriction-deficient clone (strain BK383) was identified by spotting different dilutions of Mu.K12. From spotting different dilutions of Mu-*vir* grown on strains BE72 or BK383 onto strains BE72, BE257, BE258, BK300, and BK383, it was concluded that strain BK383 is restriction deficient and modification proficient.

Phage-sensitivity tests. These were done either by the cross-streak method (16) or by spotting dilutions of phage on bacteria to be tested on φ plates. Scoring was done after overnight incubation at 42°C.

Phage assays. Phage assays were done by the soft-agar overlay technique using φ soft agar, φ plates, and exponential-phase indicator bacteria grown in φ broth. Plates were incubated overnight at 42°C before they were counted.

TABLE 1. *Bacterial strains*

Strain no.	Genotype/phenotype	Source/references
Klebsiella pneumoniae M5a1		
BK3 (synonym: UNF 9232)	*leu-2 trp-1 lys-1 his-2 nif-9 str-1*	J. Postgate (8)
BK41	(*his-kth*)Δ*30*	K. T. Shanmugam (19)
BK81	BK41, *str-201*	Spontaneous from BK41
BK111	*leu-2 trp-1 lys-1 nif-9* (*his-kth*)Δ*108 str-1*	Derivative of BK3[b]
BK202	Wild type	K. T. Shanmugam
BK203	R144*drd-3*/*met*	K. T. Shanmugam
BK277	RP4(Mu*cts61*)/BK202[a]	BE228 + BK202 → Tc^r Ap^r Km^r, TS
BK300	BK202, Mu[s]	NTG mutagenesis of BK202
BK307	BK202, (*his-kth*)Δ*201*	Spontaneous from BK202[b]
BK309	BK202, (*rtl-kth*)Δ*202*	Spontaneous from BK202[b]
BK310	BK202, (*rtl-kth*)Δ*203*	Spontaneous from BK202[b]
BK321	BK202, (*his-kth*)Δ*90*	K. T. Shanmugam
BK366	BK300, *rtl-201*::(Mu*cts61*)[c]	Mu infection of BK300
BK368	BK300, *rtl-202*::(Mu*cts61*)	Mu infection of BK300
BK373	RP41/BK309[d]	BE202 + BK309 → His$^+$, Tc^r Ap^r Km^r
BK383	BK300, *hsdR201*	Ethyl methane sulfonate mutagenesis of BK300
BK392	BK300, *trp-201*::(Mu*cts61*)	Mu infection of BK300
BK429	R144*drd-3*/BK86	BK203 + BK86 → Km^r
BK444	BK300, *nif-1001*::(Mu)	Mu infection of BK300
BK446	BK383, *nif-1002*::(Mu)	Mu infection of BK383
BK485	R144*drd-3*/BK444	BK429 + BK444 → Km^r
BK487	R144*drd-3*/BK446	BK429 + BK446 → Km^r
BK489	BK81, His$^+$	BK485 + BK81 → His$^+$ (Str^r)
BK490	BK81, His$^+$, *nif-1001*::(Mu)	BK485 + BK81 → His$^+$ (Str^r)
BK495	BK81, His$^+$	BK487 + BK81 → His$^+$ (Str^r)
BK496	BK81, His$^+$, *nif-1002*::(Mu)	BK487 + BK81 → His$^+$ (Str^r)
BK497	BK373, Rtl$^+$	BK485 + BK373 → Rtl$^+$ (Tc^r Ap^r Km^r)
BK499	BK373, Rtl$^+$, *nif-1001*::(Mu)	BK485 + BK373 → Rtl$^+$ (Tc^r Ap^r Km^r)
BK500	BK373, Rtl$^+$	BK487 + BK373 → Rtl$^+$ (Tc^r Ap^r Km^r)
BK502	BK373, Rtl$^+$, *nif-1002*::(Mu)	BK487 + BK373 → Rtl$^+$ (Tc^r Ap^r Km^r)
Escherichia coli K-12		
BE72 (synonym: C600)	*thr-1 leu-6 thi-1 supE44 lacY1 tonA21* λ$^-$	F. Jacob
BE117	*leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 sup-37 recB21 recC22 sbcB15 hsdS*	Derived from JC7623 (5) by P1 transduction[b]
BE128	F'*pro*$^+$ *lacI*::(Mu*cts62*)/(*pro-lac*)Δ, *str*^{r}	A. I. Bukhari (27)
BE202	RP41/JC5466 Trp$^-$	R. A. Dixon via K. T. Shanmugam (7)
BE228	RP4 (Mu*cts61*)/C600	M. van Montagu
BE245	C600 (Mu)	A. Toussaint
BE252	BE117, P1^r	Spontaneous from BE117
BE257	C600, *hsdR*[e]	R. Davis (4)
BE258	C600, *hsdS*	R. Davis (4)

[a] When the location of Mu insertion is not known, Mu is written in parentheses.

[b] Isolation and characterization of these strains will be published separately (manuscript in preparation).

[c] When the location of Mu insertion is known, Mu is written after the locus as *rtl-201*::(Mu*cts61*).

[d] RP41 is a plasmid belonging to the incompatibility group P conferring resistance to tetracycline (Tc), ampicillin (Ap), and kanamycin (Km) and carrying *his-nif* region of *K. pneumoniae* (7).

[e] On single-colony isolation, two types of colony morphology were observed: small smooth and medium wrinkled. Both types were *hsdR*. Strain BE257 is a smooth colony type.

Scoring of unselected markers. Unselected markers were scored as described before (15, 16). The Nif$^+$ marker was scored by colony-forming ability on nitrogen-free plates (21), and selected clones were also checked using the whole-cell acetylene reduction assay (19). Lysogens carrying temperature-inducible prophage were identified by their temperature-sensitive growth and by their ability to release infectious phage at 42°C and lyse a lawn of strain BK300 (9). Lysogens carrying wild-type Mu prophage were identified by their ability to release infectious phage and by their immunity to infection by phage Mu*cts61*.

Nomenclature. The recommendations of Demerec et al. (6), Bachmann et al. (1), and Low (12) were followed. Nomenclature used for restriction experiments has been described before (16). Ability to convert molecular nitrogen to ammonia was denoted by *nif*, and resistance to bacteriophage K3 infection was denoted by *kth*. Ability to utilize ribitol as a carbon source was denoted by *rtl* (17).

RESULTS

Transfer of Mu to *K. pneumoniae*. The first experiments were concerned with whether Mu was able to replicate in the cytoplasm of a nitrogen-fixing strain of *K. pneumoniae*. Since *K. pneumoniae* was not infected by Mu, it was necessary to introduce Mu via a plasmid harboring Mu. The release of phage would indicate that Mu is indeed able to multiply in *K. pneumoniae*. To introduce Mu, a plasmid carrying temperature-inducible Mu (RP4[Mu*cts61*]) was conjugally transferred from an *E. coli* donor to a wild-type *K. pneumoniae* M5a1 recipient, selecting for the transfer of plasmid-determined antibiotic resistance. All of the 200 progeny clones tested were found to be able to grow at 25°C but not at 42°C (temperature-sensitive growth) and to be able to release phage, at 42°C, which could grow in a Mu-sensitive *E. coli* indicator strain (BE117). One clone was purified and labeled BK277. When strain BK277 was plated on plates with strain BE117, all the colony-forming units appeared as infective centers, showing that all of the clones harbored phage.

Evidence that Mu replicates. The evidence that Mu is in fact capable of replication in *K. pneumoniae* may be summarized as follows: (i) infectious phage were released from strain BK277 (harboring Mu on a plasmid) upon thermal induction, and (ii) the phage released from *K. pneumoniae* (strain BK277) was found to be restricted by *E. coli* K-12, suggesting that the phage acquired *K. pneumoniae* modification. For this experiment, phage Mu grown by inducing the strains BE228 (*E. coli* K-12) and BK277 (*K. pneumoniae* M5a1) were assayed on restriction-deficient *E. coli* strain BE117 and on isogenic restriction-proficient strain BE83. The efficiency of plating (EOP) of phage from strain BE228 on strain BE83 was 0.72 of that on strain BE117, whereas the EOP of phage from strain BK277 on strain BE83 was 0.01 of that on strain BE117. From this we infer that Mu is able to grow in *K. pneumoniae*.

Isolation of a Mu-sensitive strain, BK300. The finding that Mu was unable to infect *K. pneumoniae* but could replicate once introduced via a plasmid suggested that Mu may be incapable of attachment to *K. pneumoniae*. Therefore, mutant strains of *K. pneumoniae* sensitive to infection by Mu were sought. Wild-type *K. pneumoniae* M5a1 was mutagenized with NTG, and the cells were allowed to segregate first in ϕ broth at 42°C and later in M9 sucrose medium at 37°C. These cells were spread on ϕ plates to give about 200 colonies/plate along with different dilutions of Mu*cts61* (to yield 10^5 to 10^7 phage/plate) induced from strain BK277. The plates were incubated overnight at 42°C. About 0.5% of the colonies were nibbled (14). From these, a Mu-sensitive clone was isolated (strain BK300).

In addition to this clone, a survey of our culture collection revealed that several other strains of *K. pneumoniae* carrying deletions of the histidine region were sensitive to infection by Mu. The EOP of Mu*cts61* obtained from strain BK368 on strains BK41, BK111, and BK310 varied from 6×10^{-3} to 1×10^{-1} (Table 2).

Lysates of Mu, Mu*cts62* and Mu*vir*, made on *E. coli* K-12 were found to plate on strain BK383, a restriction-deficient derivative of Mu-sensitive *K. pneumoniae* strain BK300, with an EOP of about 2×10^{-1}. From this, we conclude that strain BK300 is sensitive to different mutant strains of Mu.

Isolation and characterization of Mu-induced *nif* mutations. In *E. coli* K-12, Mu is able to insert itself at random, inducing mutations (3, 11, 24). The availability of Mu-sensitive strains of *K. pneumoniae* provides us with a new tool for generation of a variety of mutations. Mutations were isolated from Mu-infected cultures as described in Materials and Methods. A total of at least 15 different mutations were isolated from Mu-infected cultures (Table 3).

When Mu-induced mutations are transduced to prototrophy, the recombinants are found to have lost the prophage Mu (2, 3, 10, 11, 25). This indicates that the mutation is a result of Mu insertion. Since strain BK300 was insensitive to P1 infection, we used R144*drd-3*-mediated chromosome mobilization to study this

TABLE 2. *Deletions of the histidine region result in partial sensitivity to phage Mu.*

Phage	EOP on strain:			
	BK300, Mu[a]	BK41, (*his-kth*)Δ30[a]	BK111, (*his-kth*)Δ108[a]	BK310, (*his-kth*)Δ203[a]
Mu	1	4.3×10^{-6}	$\sim 1 \times 10^{-7}$	$\sim 1 \times 10^{-7}$
Muc*ts61*[b]	1	1.4×10^{-1}	6.4×10^{-3}	8.2×10^{-3}

[a] Relevant property.
[b] Phage was obtained by induction of strain BK368.

TABLE 3. *Mutations isolated from Mu-infected cultures*

Strain		Penicillin enrichment	Total no. of clones examined	No. of different mutants	Mutant types isolated
Bacteria	Phage				
BK300	Muc*ts61*	Yes	NA[a]	4	*trp, ile*, vitamins, and complex
	Muc*ts61*	Yes	NA	2	*rtl* and *gat*
	Muc*ts61*	Yes	NA	2	*rtl* and *gat*
	Mu	Yes	NA	1	*nif*
	Mu	Yes	NA	8	*trp, his, leu, arg, gly, met*, and complex
	Muc*ts61*	No	~1,500	5	*trp, met, thi, pro*, and complex
	Muc*ts61*	No	~2,000	0	None
BK383	Muc*ts61*	No	~2,000	2	*met* and complex
	Muc*ts61*	No	~3,000	1	*lys*
	Mu	Yes	NA	1	*nif*

[a] NA, Not applicable.

linkage between mutant phenotype and Mu. A total of five independent Mu-induced mutations (two *nif*, one *trp*, and two *rtl* mutants) were genetically analyzed in seven crosses (Table 4), and all five mutations were found to be linked to the presence of Mu. Crosses 1, 2, 3, and 4 involved transfer of Mu-induced mutations as an unselected marker, selecting either for His^+ (Str^r) (crosses 1 and 2) or for Rtl^+ (Tc^r Ap^r Km^r) (crosses 3 and 4). Crosses 5, 6, and 7 involved selections of Mu-induced mutations to prototrophy. In all cases, the co-transfer frequency between Mu and the mutation was at least 85% or greater. The precision of this analysis is limited by the fact that prototrophic recombinants were generated by R144*drd-3*-mediated chromosome mobilization (8). In crosses 1 and 2, about 2 to 10% of the His^+ (Str^r) progeny was Nif^-, and all the Nif^- progeny was lysogenic for Mu. A large number of the *nif* mutations have been found to co-transduce with the *hisD* locus (20, 22). Co-transfer between *his* and *nif* observed in crosses 1 and 2 indicates that these two *nif* mutations are in the *nif* cluster near the *his* region (operationally, we define a *nif* mutation as lying in the *nif* cluster near the *his* region if the two mutations are shown to be linked either by P1 transduction or by R144*drd-3*-mediated chromosome mobilization). In crosses 3 and 4, about 0.6 to 3% of the Rtl^+ (Tc^r Ap^r Km^r) progeny was Nif^-, and all the Nif^- progeny was lysogenic for Mu. In crosses 6 and 7, more than 90% of the progeny had acquired the donor phenotype, Nif^-, and of these, more than 93% had lost the prophage Mu. We interpret this to indicate that the *nif-9* mutation in strain BK429 is linked to *rtl* marker. The presence of Mu in a small fraction of the Rtl^+ progeny probably represents relysogenization of the Rtl^+ progeny by phage Mu present in the mating mixture. The frequency of co-transfer of *rtl* with *nif* is much lower in crosses 3 and 4 than in crosses 6 and 7. We suggest two explanations for this: (i) crosses 1, 2, 3, and 4 involved a donor lysogenic for Mu, whereas in crosses 6 and 7, the recipient was lysogenic for Mu. Entry of Mu into a nonlysogenic cell is likely to lead to zygotic induction, thus decreasing the yield of progeny that has received Mu or markers close by; (ii) crosses 3 and 4 are complicated by the fact that the recipient strain, BK373, is deleted for (*rtl-kth*) and carries the RP41 plasmid. The Rtl^+ phenotype can be generated by recombinational events either with the chromosome or with the plasmid. However, generation of the nif^- phenotype requires a recombinational event only with the plasmid.

Characterization of the primary isolations of Mu-induced *nif* mutations, the His^+ (Str^r) derivatives and the Rtl^+ (Tc^r Ap^r Km^r) derivatives, is presented in Table 5. This table shows correlation of inability to grow on N_2, lack of

TABLE 4. *Genetic analysis of Mu-induced mutations*[a]

Cross no.	Donor	Recipient	Selected marker	Recombination frequency	Inheritance of unselected markers[b]				Co-transfer frequency between:	
					Nif⁻		Nif⁺		Mu and the mutation	Selected marker and *nif* mutation
					Mu	0	Mu	0		
1	BK485 (*nif-1001*, Mu)[c]	BK81 (*hisΔ30 str*)	His⁺ (Str^r)	~4 × 10⁻⁶	2[d]	0	ND[e]	78	2/2	2/80
2	BK487 (*nif-1002*, Mu)	BK81 (*hisΔ30 str*)	His⁺ (Str^r)	~2 × 10⁻⁶	4[d]	0	ND[e]	32	4/4	4/36
3	BK485 (*nif-1001*, Mu)	BK373 (*rtlΔ202* Tc^r Ap^r Km^r)	Rtl⁺ (Tc^r Ap^r Km^r)	~1 × 10⁻⁵	2[d]	0	ND[e]	297	2/2	2/299
4	BK487 (*nif-1002*, Mu)	BK373 (*rtlΔ202* Tc^r Ap^r Km^r)	Rtl⁺ (Tc^r Ap^r Km^r)	~1 × 10⁻⁵	5[d]	0	ND[e]	159	5/5	5/164
5	BK203 (*nif*⁺)	BK392 (*trp-201*, Mu)	Trp⁺	~9 × 10⁻⁵	—[f]	—[f]	1	128	128/129	
6	BK429 (*nif-9*)	BK366 (*rtl-201*, Mu)	Rtl⁺	~3 × 10⁻⁷	7	53	2	3	56/65	60/65
7	BK429 (*nif-9*)	BK368 (*rtl-202*, Mu)	Rtl⁺	~2 × 10⁻⁷	2	40	3	0	40/45	42/45

[a] Details are given in the text.
[b] Mu represents Mu lysogenic condition, and 0 represents nonlysogenic condition.
[c] Relevant markers are listed in parenthesis.
[d] These clones were identified by their inability to grow on molecular nitrogen. All of them were unable to reduce acetylene at the whole cell level.
[e] Nif⁺ clones were not checked for the presence of Mu.
[f] This class does not exist.

TABLE 5. *Characterization of Nif⁻ mutations induced by Mu*

Strain	Growth on N_2[a]	μmol of C_2H_2 reduced/h per mg of protein	Release of Mu (plaque-forming units/ml)	Immunity to Mu	Str^r	Tc^r Ap^r Km^r
Parents						
BK300	+	2.41	<10⁻⁶	—[b]	s	s
BK383	+	1.23	<10⁻⁶	—	s	s
Nif⁻ mutants						
BK444	—	0.18	~5 × 10⁻⁴	+	s	s
BK446	—	<0.01	~5 × 10⁻⁴	+	s	s
Nif⁻ mutants carrying R144*drd-3*						
BK485	—	0.08	~5 × 10⁻⁴	+	s	s
BK487	—	0.01	~5 × 10⁻⁴	+	s	s
Recipients						
BK81	+	2.31	<10⁻⁶	ND[c]	r	s
BK373	+	1.17	<10⁻⁶	ND	s	r
BK81 His⁺ (Str^r) progeny						
BK489	+	2.41	<10⁻⁶	ND	r	s
BK490	—	0.04	~5 × 10⁻⁴	ND	r	s
BK495	+	1.99	<10⁻⁶	ND	r	s
BK496	—	0.02	~5 × 10⁻⁴	ND	r	s
BK373 Rtl⁺ (Tc^r Ap^r Km^r) progeny						
BK497	+	2.21	<10⁻⁶	ND	s	r
BK499	—	0.03	~5 × 10⁻⁴	ND	s	r
BK500	+	1.58	<10⁻⁶	ND	s	r
BK502	—	<0.01	~10⁻⁵	ND	s	r

[a] +, Ability to grow to N_2; —, inability to grow on N_2.
[b] —, Sensitive to Mu infection; +, immune to Mu infection.
[c] ND, Not determined.

whole-cell acetylene-reducing activity, the presence of Mu, and immunity to Mu.

DISCUSSION

The method used to isolate the Mu-sensitive strain involved: (i) showing that the *K. pneumoniae* cytoplasm supports the growth of Mu, and (ii) utilizing Mu modified with M5a1 specificity. These general approaches may be exploited in increasing the host range of useful temperate phages for other enterobacteria.

All Mu-induced mutations that were analyzed have been found to co-transfer with Mu at a high frequency, indicating that insertion of Mu has resulted in the mutant phenotype. However, it is not clear whether the Mu-induced mutants are monolysogenic or polylysogenic (tandem or clustered). The precision of this analysis is limited by the fact that chromosomal segments mobilized by R144*drd-3* are longer than those transferred in P1 transduction (8; unpublished observations). The genetic analysis suggests that both Mu and *nif* mutations map in the *nif* cluster near the histidine region. The Nif$^-$ phenotype could easily result from several nonstructural gene alterations, and our data are not sufficient to prove that the *nif* mutations resulted from the insertion of Mu into *nif* structural genes.

With the availability of Mu-sensitive *K. pneumoniae* strains, it will be possible to use this system to study the *nif* operon structure by analyzing the polarity of Mu-induced *nif* mutations either biochemically or genetically. However, before the polarity studies can be properly interpreted, it is necessary to show that the mutant phenotype is a result of the insertion of a single Mu prophage in a *nif* structural gene. We are also exploring the possibilities of using Mu to generate deletions and to isolate Hfr strains in *K. pneumoniae*.

Utilization of Mu in the isolation of deletions in *K. pneumoniae* has been reported (M. Bachhuber, T. Malavich, and M. Howe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, H77, p. 108).

In summary, our work has described: (i) a method of sensitizing *K. pneumoniae* M5a1 to a temperate phage, Mu; (ii) Mu-induced mutations in *K. pneumoniae*; and (iii) linkage between *rtl* and *nif* loci in *K. pneumoniae*.

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