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Use of Bacteriophage Mu to Isolate Deletions in the *his-nif* Region of *Klebsiella pneumoniae*

MARY BACHHUBER, WINSTON J. BRILL, AND MARTHA M. HOWE*

Department of Bacteriology and Center for Studies of Nitrogen Fixation, University of Wisconsin, Madison, Wisconsin 53706*

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Klebsiella pneumoniae M5a1 is naturally resistant to infection by bacteriophage Mu. Mutants of *K. pneumoniae* sensitive to Mu infection were isolated and found to support both lytic and lysogenic development of Mu. *K. pneumoniae* lysogens containing a heat-inducible Mu prophage integrated in *his* were isolated. Strains carrying deletions extending from *his* into *nif* were obtained after heat treatment of these lysogens. Such deletions should be useful for determining the map order and cistronic organization of the *nif* genes.

When *Escherichia coli* K-12 is infected with bacteriophage Mu, some of the resulting lysogens contain mutations that map at various sites on the chromosome (4). These mutations, which are genetically inseparable from a Mu prophage, are caused by integration of Mu into a gene.

Lysogens containing a heat-inducible Mu prophage die when grown at high temperature, due to induction of phage development (2). Some of the heat-resistant survivors of induction are deleted for the immunity and killing functions of Mu. Since these deletions often extend into nearby bacterial deoxyribonucleic acid, induction of appropriately located Mu prophages can be used to generate deletions in specific regions of the chromosome.

A number of Nif$^-$ (unable to fix N_2) mutant strains of *Klebsiella pneumoniae* have been isolated and characterized (7). Previous mapping of these *nif* mutations was accomplished using three-factor crosses by P1 transduction, with a nearby *his* mutation (1, 10) being used as the third marker (7, 10). To facilitate further mapping of *nif* mutations by the technique of deletion mapping, we isolated Mu-induced deletion mutations extending from *his* into the *nif* region of *K. pneumoniae*.

(Part of this work was 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, SB, SBPM, and SM were given previously (2, 3). Minimal medium was also described previously (7, 12). TCM contains 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5), 0.01 M $CaCl_2$, and 0.01 M $MgSO_4$. LC broth (pH 7.0) contains 1% tryptone, 0.5% yeast extract, and 1% sodium chloride. LC-Ca-Mg is LC supplemented with 2.5×10^{-3} M $CaCl_2$ and 2.5×10^{-3} M $MgCl_2$. Soft agar and LC plates contain LC broth supplemented with 0.4 and 1.0% agar, respectively. P1-dil contains (per liter) 10 g of tryptone and 2 g of $MgCl_2$. B-dil contains (per liter) 2 g of $MgCl_2$ and 2 g of $CaCl_2$.

Bacterial strains. *K. pneumoniae* M5a1 was obtained from P. W. Wilson. Strains containing *nif* mutations 4026 through 4116 were described by St. John et al. (7). Strain MH812 (*thr leu met lac* su_{II}^+ *hsdM hsdR*) is strain 921, a restriction-modification-deficient mutant of *E. coli* K-12, obtained from W. Arber. Strains M107 (F$^-$ *lac* su_I^+ *strA*) and QD5003 (*mel* su_{III}^+) are *E. coli* K-12 strains used for growing and titering Mu (2). Strain MH125 (*araD leu*::Mu *cts*61) is an *E. coli* K-12 strain containing a heat-inducible prophage integrated in *leu* (2).

Bacteriophage strains. Mu *cts*61 and Mu *cts*62 are heat-inducible mutants of Mu (2). Mu *c*25 is a clear mutant of Mu isolated without mutagenesis from a lysate of wild-type Mu by M. Howe. P1*kc* was obtained from W. H. McClain.

Assays for Mu lysogeny. (i) Cross-streak. Colonies were picked with a sterile toothpick and cross-streaked against a lysate of Mu *cts*61 or Mu *c*25 at 10^9 phage per ml on thick LC plates (2). Immune or resistant colonies were unaffected, whereas sensitive colonies were lysed at their intersection with the phage lysate.

(ii) Phage release. Colonies were transferred with toothpicks or replicated onto LC plates overlaid with lawns containing 10^8 cells of strain MH812 suspended in soft agar (2). After overnight incubation, lysogens showed a halo of lysis around the colony.

Preparation of lysates. Preparation of Mu lysates by heat induction was done as described previously (2) except that Pb^{2+} was omitted.

To prepare Mu lysates by infection, 0.2-ml volumes of cells growing exponentially at 10^8/ml in LC-Ca-Mg at 37°C were mixed with two fresh Mu plaques. After 20 min at 37°C the mixture was diluted with 7.5 ml of a similar medium and shaken at

37°C until lysis occurred at 3 to 5 h. The lysate was chilled, a few drops of chloroform were added, and the cell debris was removed by centrifugation at 15,000 rpm for 30 min in a Sorvall SS34 rotor. The lysate supernatant was stored at 4°C.

P1 lysates were prepared by taking 2.5×10^7 cells growing exponentially in LC, mixing them with 10^7 P1 in a volume of 0.35 ml, incubating the mixture at 37°C for 20 min, and plating the mixture in 2.5 ml of soft agar containing 2×10^{-4} M thymine, 2×10^{-3} M $CaCl_2$, and 0.1% glucose on LC plates. After 12 to 18 h at 37°C, the soft-agar layer was scraped off the plates, treated with chloroform, and centrifuged at 4,000 rpm for 15 min in a Sorvall SS34 rotor. The supernatant solution was collected and stored at 4°C.

Procedures for titration of Mu (2) and P1 (11) were described previously. When Mu was titered on *K. pneumoniae*, 2.5×10^{-3} M $CaCl_2$ and 2.5×10^{-3} M $MgSO_4$ were added to the soft-agar overlay.

P1 transduction. P1 transductions were done as described by Wolf et al. (11).

Assay for Mu adsorption. Cells were grown in LB containing 10^{-3} M $CaCl_2$ and 10^{-3} M $MgSO_4$ to a density of 10^9/ml, pelleted by centrifugation, and suspended in 0.10 volume of TCM containing 100 μg of chloramphenicol per ml. After incubation for 15 min at 37°C, a lysate of Mu *cts*62 stored in SBPM was added at a multiplicity of 0.04. Portions were removed at various times, diluted into SM containing chloroform, and titered on strain QD5003. Adsorption rates were corrected for spontaneous phage inactivation under the assay conditions as determined in assay mixtures lacking cells or containing Mu^r cells.

Penicillin enrichment for His auxotrophs. The Mu-infected culture of strain UN729 was pelleted, washed, diluted into minimal medium, and grown to approximately 2×10^8 cells per ml. One cycle of penicillin selection was carried out at 32°C as described by Roth (6).

Linkage of Mu prophage to *his* mutations. The recipient strains were grown in LC-Ca-Mg to a density of 3×10^8 cells per ml, pelleted by centrifugation, and suspended to 6×10^8 cells per ml in B-dil. These cultures were infected at a multiplicity of one with P1 grown on strain UN729 and incubated at 32°C for 20 min. Portions containing 6×10^7 cells were spread on minimal plates without histidine to select His^+ transductants. After 2 days at 32°C, 15 transductants from each strain were purified, and four clones from each were tested for Mu lysogeny by their ability to release phage capable of growth on a lawn of strain MH812 at 42°C.

Isolation of Mu-induced deletions. Lysogens containing Mu *cts*61 integrated in *his* were grown at 32°C in SB containing 10^{-3} M $CaCl_2$ and 10^{-3} M $MgSO_4$. When the cells reached a density of 10^8 per ml, an equal volume of medium at 60°C was added and the cultures were shifted to 42°C. Mu-specific antiserum was added to a final K value of 11.8 min^{-1}, and the cultures were grown at 42°C for 4 h. Cells and cell debris were pelleted by centrifugation, washed and suspended in B-dil, and streaked at several dilutions on LC plates, which were incubated overnight at 42°C. The survivors were tested for Mu sensitivity by cross-streaking.

Assay of Nif activity. Assays of Nif activity by acetylene reduction (9) and by growth on nitrogen-free media (7) were described previously.

RESULTS

Isolation of Mu-sensitive *K. pneumoniae*. *K. pneumoniae* M5a1 is naturally resistant to infection by bacteriophage Mu. In light of the observation that some strains of *Salmonella typhimurium* resistant to P22 are sensitive to P1 (5), it seemed possible that mutants of *K. pneumoniae* resistant to *K. pneumoniae*-specific phages might have altered cell walls that would allow adsorption of Mu. Six virulent phage capable of growth on *K. pneumoniae* were isolated from filter-sterilized portions of soil or sewage suspended in P1 diluent. Mutant strains of *K. pneumoniae* resistant to each phage were selected as survivors of phage infection and were tested for their sensitivity to Mu by cross-streaking them against a high-titer lysate of Mu *cts*61 grown in *E. coli*. Four of the six phages produced Mu-sensitive strains at frequencies of 10 to 50% among the survivors. Although the exact nature of the mutations to Mu sensitivity has not been determined, in the four strains tested they were 55% linked to *his* by P1 transduction and therefore might be in genes analogous to the *rfb* genes of *S. typhimurium* (8).

Lysates of Mu *cts*61 and Mu *c*25 grown on one of these Mu-sensitive *K. pneumoniae* strains were used for all subsequent experiments. When Mu lysates were titered on these strains, the efficiency of plating varied from 10^{-2} to 10^{-6} relative to plating on strain MH812, a restriction-deficient strain of *E. coli* K-12. The strains that gave the highest efficiency of plating for Mu were all P1 resistant, but some of the other strains retained some sensitivity to P1.

Strain UN729 was chosen for further use because it was moderately sensitive to both Mu and P1. Lysates of Mu could be grown on strain UN729 in liquid medium; however, the plating efficiency on agar was quite low. This is probably due to slow adsorption, since under conditions allowing 95% adsorption of Mu to *E. coli* there was less than 10% adsorption of Mu to strain UN729. Therefore, it appears that these strains possess a receptor site that is recognized inefficiently by Mu. Mutants of Mu able to plaque more efficiently on these strains have been isolated but not characterized.

Lytic and lysogenic development of Mu in *K. pneumoniae*. The time course of phage production after heat induction of Mu *cts*61 lysogens appeared to be similar for both *E. coli* and

K. pneumoniae strains, except that phage production in *K. pneumoniae* was retarded by approximately 10 min relative to that of *E. coli* (Fig. 1). The delayed lysis time was observed in all *K. pneumoniae* strains tested even when induction was performed at different cell densities. The burst size of Mu was 50 to 60 plaque-forming units per cell from both strains.

The frequency of lysogenization and Mu-induced mutation in *K. pneumoniae* UN729 is shown in Table 1. When strain UN729 was infected with Mu *cts*61 and grown to saturation, the resulting culture contained less than 1% lysogens and, as expected from the low frequency of lysogens, exhibited no detectable increase in the frequency of nalidixic acid-resistant mutants. This culture was then infected with Mu *c*25 to kill nonlysogens and enrich for lysogens by their immunity to superinfection. In the population surviving Mu *c*25 infection, the frequency of lysogens increased to 44%, and

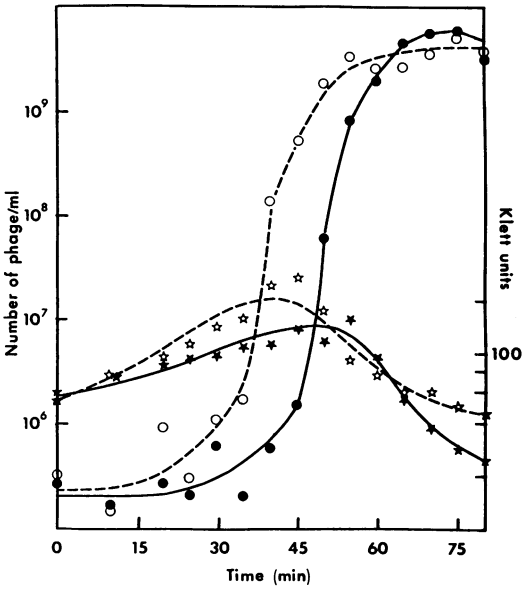

FIG. 1. *Heat induction of E. coli and K. pneumoniae lysogens. Mu cts61 lysogens of strain UN729 (K. pneumoniae) and strain MH125 (E. coli) were grown at 30°C in SB supplemented with 2.5 × 10^{-3} M $CaCl_2$ and 2.5 × 10^{-3} M $MgSO_4$ to a density of 2 × 10^8 cells per ml. At time zero, equal volumes of medium at 60°C were added, and the cultures were grown thereafter at 42°C. Portions were removed at the times indicated, diluted into LC containing chloroform, and titered for free phage on strain MH812. Cell density was monitored with a Klett colorimeter. Symbols: ○, phage production from strain MH125; ●, phage production from strain UN729 (Mu cts61); ☆, cell density for strain MH125; ★, cell density for strain UN729 (Mu cts61).*

TABLE 1. *Mutagenesis of K. pneumoniae by Mu*[a]

Culture	Frequency of lysogens (%)	Frequency of Nal^r	Frequency of Nal^r that are lysogens (%)
Uninfected		3.2 × 10^{-6}	
Infected, Mu *cts*	0.23	1.1 × 10^{-6}	3
Infected, Mu *cts*, Mu *c*25	44	8.5 × 10^{-5}	95

[a] Strain UN729 was grown in LC-Ca-Mg to a density of 10^8 cells per ml. A portion was infected at a multiplicity of 10 with a lysate of Mu *cts*61 previously grown by heat induction from strain UN729 (Mu *cts*61). After 18 h at 30°C the cells were pelleted by centrifugation and washed and suspended in an equal volume of B-dil. A portion of these cells was diluted into 5 ml of LC-Ca-Mg, grown to a density of 2 × 10^8 cells per ml, mixed with two fresh plaques of Mu *c*25 grown on strain UN729, diluted 40-fold with LC-Ca-Mg, and shaken at 32°C for 5 h. The cells were centrifuged and washed and suspended in B-dil. Portions of each culture were diluted and plated on LC plates containing 25 μg of nalidixic acid per ml to score for mutagenesis and on LC to determine the viable count. The frequency of lysogens was determined by phage release.

the frequency of nalidixic acid-resistant mutants, presumably caused by Mu integration, increased 77-fold. Therefore, although the initial frequencies of lysogens and Mu-induced mutations in the cultures of strain UN729 were low, they could be increased, by selection for Mu immunity, to levels high enough to enable the isolation of strains containing Mu integrated in specific sites of interest.

The above-mentioned results differ from those observed in *E. coli* in which the population surviving Mu infection contained approximately 50% lysogens and exhibited a 50- to 100-fold increase over the spontaneous mutation frequency in a single gene (4). The lower frequency of lysogens in cultures of strain UN729 is probably due to poor adsorption of Mu to strain UN729 rather than to a difference in the ability of Mu to establish lysogeny in *K. pneumoniae*. Infection of other *K. pneumoniae* strains more sensitive to Mu has resulted in populations exhibiting frequencies of lysogenization and mutagenesis comparable to those observed in *E. coli*.

Isolation of Mu-induced *his* mutations. Lysogens containing Mu *cts*61 integrated in *his* near *nif* were obtained after penicillin treatment of the enriched lysogenic population of strain UN729. After penicillin treatment, the culture contained 4% His auxotrophs, all of which were lysogenic for Mu as demonstrated by their ability to release phage. To confirm

that these lysogens contained Mu inserted at the *his* locus, the strains were transduced to His$^+$ by using phage P1 grown on strain UN729, the His$^+$ nonlysogenic parent. His$^+$ transductants were purified and tested for the presence of Mu prophage by testing their ability to release phage. In 16 out of 18 strains tested, transduction to His$^+$ resulted in curing of the Mu prophage, thus demonstrating that Mu was integrated in *his*. The remaining two strains did not lose the Mu prophage when transduced to His$^+$. These strains might be double lysogens containing one Mu prophage in *his* and a second elsewhere, or they might contain a spontaneous *his* mutation and an unlinked Mu prophage.

Selection for deletions. Lysogens of the heat-inducible prophage Mu *cts* die when grown at 42°C, whereas mutants unable to express Mu killing functions can survive. Survivors include lysogens containing defective Mu prophages that are still immune to superinfection and lysogens that are deleted for at least the killing and immunity functions of Mu and nearby host deoxyribonucleic acid. These deleted lysogens are sensitive to superinfection by Mu.

Cultures of the lysogens containing Mu *cts*61 integrated in *his* were grown at 42°C for 4 h in the presence of Mu-specific antiserum to prevent re-infection of the Mu-sensitive deletion strains by the free phage produced. The survivors were then tested for Mu sensitivity by cross-streaking against Mu *c*25. Seven Mu-sensitive survivors (strains UN901 through UN907), isolated from independent heat selections, were tested for Nif activity by their ability to reduce acetylene in vivo and by their ability to grow anaerobically on nitrogen-free media. Four of these strains arising from a single lysogenic strain, UN878, were found to be Nif$^-$.

The extent of these presumptive deletions into *nif* was mapped by P1 transduction. P1 was grown on the four His$^-$ Nif$^-$ strains and on a control His$^-$ Nif$^+$ strain and was used to transduce to Nif$^+$ a series of strains containing *nif* point mutations that had been mapped previously by three-factor crosses (7). The Nif$^+$ control strain UN903 gave Nif$^+$ transductants with all recipients (Table 2). The four presumptive deletion strains transduce only some of the Nif$^-$ strains to Nif$^+$. The transduction pattern obtained demonstrates that these strains do contain deletions within *nif* and confirms the order of *nif* mutations obtained previously by three-factor crosses (7).

DISCUSSION

Strains of *K. pneumoniae* sensitive to bacteriophage Mu were isolated from a collection of mutants resistant to *Klebsiella*-specific phages obtained from nature. Although the efficiency of adsorption of Mu to these strains varied and was often low, Mu could undergo both lytic and lysogenic development in them. The burst size and kinetics of phage development resulting from heat induction of Mu *cts*61 lysogens appeared to be similar in *E. coli* and *K. pneumoniae* except that development was delayed by 10 min in *K. pneumoniae*. The frequency of lysogenization and mutagenesis by Mu *cts*61 was lower in *K. pneumoniae* UN729 than in *E. coli*, but a lysogenic population could be obtained by killing the nonlysogens by infection with Mu *c*25. The mutation frequency within the resulting lysogenic population was high, as expected, since Mu prophage integration often causes mutations. Other Mu-sensitive *K. pneumoniae* strains gave higher frequencies of lysogenization and mutagenesis.

Lysogens containing Mu *cts*61 integrated in the *his* genes were isolated. When these strains were grown at 42°C, development of Mu was induced, resulting in phage production and cell death. Among the survivors of heat induction

TABLE 2. *Determination of the extent of Mu-induced deletions into nif by P1 transduction*[a]

Strain no.	Nif phenotype	*nif-4106*	*nif-4066*	*nif-4026*	*nif-4113*	*nif-4083*	*nif-4116*
		MoCo	e$^-$ transport	I	I	II	II
UN903	Nif$^+$	35[b]	990	164	524	1,096	109
UN901	Nif$^-$	0	0	0	50	106	45
UN902	Nif$^-$	0	0	0	151	238	48
UN904	Nif$^-$	0	0	0	28	97	20
UN906	Nif$^-$	0	0	103	143	300	70

[a] Two serial plate lysates of P1 were grown on *K. pneumoniae* strains UN901 through UN906 and used to transduce the recipient strains containing mutations *nif-4026* through *nif-4116* to Nif$^+$. The Nif activity affected by each mutation, which is given below the mutation number, was described in more detail by St. John et al. (7). Cells (3×10^8) infected with P1 at a multiplicity of 1.5 were plated on nitrogen-free medium and incubated anaerobically for 6 days at 30°C.

[b] The numbers given in the table refer to the number of Nif$^+$ transductants per plate. There were never more than two colonies per plate on the uninfected control plates.

were strains that were Nif⁻ (unable to fix N_2) and no longer immune to superinfection by Mu. These strains carry deletions that remove varying amounts of the *nif* genes. By using similar techniques we also obtained *nif* deletions in a plasmid containing the *nif* genes (Bachhuber et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976).

Bacteriophage Mu has proven to be a powerful genetic tool for the study of *E. coli* (4). It is particularly useful for: (i) the isolation of polar, nonreverting mutations due to prophage integration; (ii) the selection of deletion mutations; (iii) the integration of episomic elements into the chromosome; and (iv) the transposition of genes onto episomes. The ability of Mu to lysogenize and to develop lytically in Mu-sensitive mutants of *K. pneumoniae* should allow the application of these powerful genetic techniques to the study of *K. pneumoniae* and should greatly simplify genetic analyses in this strain.

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