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Genetic Characterization of Mu-Like Bacteriophage D108

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Infection of *Escherichia coli* by bacteriophage D108 was shown to result in the generation of apparently random chromosomal mutations. Approximately 1% of the cells lysogenized by D108, as with Mu, acquired new auxotrophic mutations. D108-induced mutations were nonreverting and were most probably the result of insertion of the D108 genome into regions of genetic function. D108 and Mu shared many similar properties but were heteroimmune and had different host ranges. Lytic infections of Mu lysogens with D108 and D108 lysogens with Mu resulted in 100-fold increases in release of phage with prophage markers over those due to spontaneous induction. Phenotypic mixing was common, with most phage carrying the prophage immunity being packaged in particles with the host range of the superinfecting phage. A fraction of the superinfecting phage genomes were, however, packaged in particles with the prophage-specified host range. Although 10% of the prophage progeny were D108-Mu genetic hybrids, superinfecting phage-induced release of the prophage with reciprocal phenotypic mixing occurred in *recA* hosts, in which the frequency of D108-Mu genetic hybrids was reduced 100-fold.

D108 is a temperate bacteriophage of *Escherichia coli* which was initially isolated by Mise (15) as a generalized transducing phage with properties different from P1. D108 was reported to transduce chromosomal markers at a frequency of 10^{-6} to 10^{-8} per phage and plasmid markers at a frequency of 10^{-9} to 10^{-10}. These values are somewhat lower than those for P1 but are similar to the values reported by Howe (10) for transduction of chromosomal markers by bacteriophage Mu. The size of the D108 transducing fragment is smaller than that of P1, as evident from reduced cotransduction frequencies of nearby *E. coli* genetic markers when determined with D108 and from the failure of D108 to transduce a large (~6×10^7 daltons) drug resistance plasmid in its entirety (15). Howe (10) has noted a similar reduction in cotransduction frequencies, as compared with P1, when using Mu. It has since been shown that the molecular weights of the Mu and D108 genomes (and, by inference, the sizes of potential transducing fragments) are quite similar (G. Gill, R. Hull, and R. Curtiss III, Abstr. Cold Spring Harbor Annu. Phage Meet. X:51, 1977).

The physical properties of the D108 phage particle as described by Mise (15) and those of Mu (21) are also quite similar. D108 and Mu have heads and contractile tail sheaths of similar dimensions. They both require calcium for growth and are unstable when stored in buffered saline or bacterial growth media for extended periods of time.

Because of these similarities and similarities we have observed between the physical structures of the D108 and Mu phage genomes (manuscript in preparation), we sought to determine whether Mu and D108 might share other characteristics. Of particular interest was the possibility that D108 might possess mutator properties similar to those described for Mu (2, 4, 5, 18). We also explored the immunity and host range relationships between the two phages. Finally, we undertook studies to identify any interaction between Mu and D108 which might occur when lysogens of one are infected with the other.

In this paper we present experiments which show that D108 is a mutator phage and that its mutator properties are similar to those described for Mu. D108 and Mu are shown to be heteroimmune and to have somewhat different host ranges. Release of Mu from Mu lysogens infected with D108 and of D108 from D108 lysogens infected with Mu was found to be increased at least 100-fold over spontaneous levels of prophage induction.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. All bacterial strains used in this study are *E. coli* K-12 derivatives and are listed in Table 1. D108 *cts10* is a thermoinducible mutant of D108 isolated after induction

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TABLE 1. *E. coli K-12 derivatives used*

Strain	Characteristics[a]	Source (reference)
χ289	F⁻ *supE42* λ⁻ T3ʳ	(7)
χ1753	F⁻ *tsx-63 supE42* λ⁻ *his-53 lysA32 T3ʳ xyl-14 arg-65*	From χ289
χ1854	*thr-1 leu-6 thi-1 supE44 lacY1 tonA21* λ⁻ (D108) (S-a)	M. Inoue

[a] χ289 was derived from W1485, and χ1854 was from C600.

of χ1854 with UV light. Phage Mu-, D108-, and P1-resistant mutants were isolated by using Mu *cts62*, D108 *cts10*, and P1*vir*, respectively, and were subsequently tested to confirm that they do not adsorb the respective phage. Bacteriophages Mu and Mu *cts62* were kindly provided by A. I. Bukhari and M. Howe, respectively; P1*vir*, P1*kc*, and P1L4 were obtained from our laboratory stocks.

Media. L broth, which was used for routine bacterial cultivation, contains (per liter): 5 g of yeast extract, 10 g of tryptone, 5 g of NaCl, and 0.1% glucose and was adjusted to pH 7.0 with NaOH (12). Plate lysates of phage were prepared on TYMC plates, which contain (per liter): 10 g of Trypticase peptone (Baltimore Biological Laboratory), 5 g of yeast extract, 5 g of NaCl, 8 g of Difco agar, 2.5 mM $CaCl_2$, and 2.5 mM $MgSO_4$. Soft agar used was the same as TYMC but with only 0.6% agar. Superbroth plus 3 mM $MgSO_4$ (SBM) (13) was used in preparing lysates of thermoinducible phage and contains (per liter): 20 g of yeast extract, 32 g of tryptone, 5 g of NaCl, 5 ml of 1 N NaOH, and 3 mM $MgSO_4$. ML and Davis-Mingioli minimal salts media and the concentrations of various growth supplements used have been described elsewhere (7, 9).

Phage propagation. Lysates of D108 *cts10* and Mu *cts62* were prepared by thermoinduction of χ289 (D108 *cts10*) and χ289 (Mu *cts62*), respectively. Overnight cultures were diluted 20-fold into SBM and allowed to grow at 30°C with moderate aeration to a cell density of 2×10^8 to 4×10^8/ml. They were then transferred to a 42°C shaking water bath and further incubated with heavy aeration until lysis was apparent (usually about 1 h). D108 *cts10* lysates prepared in this fashion usually contained about 10^{10} PFU/ml. The confluent plate lysis procedure described by Lennox (12), starting with phage from a single isolated plaque, was used to prepare Mu, P1, and D108 lysates. Titers of D108 lysates prepared by confluent plate lysis were typically 10^{11} PFU/ml.

D108 was initially isolated from nature by Mise (15) after induction of a mixed *E. coli* culture with the radiomimetic agent mitomycin C. In addition, D108 was found to be UV inducible in a χ1854 lysogen (M. Inoue, personal communication). D108 in χ289 (D108) was found not to be UV inducible, however, and the confluent plate lysis procedure was therefore used.

Single-step growth kinetics. Cells from an exponentially growing culture were collected by centrifugation and suspended in SBM broth plus 2.5 mM $CaCl_2$ at a titer of 10^9/ml. This culture was preincubated for 2 min with 1 mM potassium cyanide and then infected with phage at a multiplicity of 0.1. After 15 min of further incubation at 37°C, during which greater than 95% of the phage adsorbed, a sample was diluted 10^5-fold into prewarmed SBM broth plus 2.5 mM $CaCl_2$ and incubated at 42°C with vigorous aeration. Samples were withdrawn at various times, diluted appropriately, and plated for infective centers on TYMC agar. χ289 (Mu) was used as the D108-sensitive indicator bacterium.

Mutagenesis with D108. The method described by Boram and Abelson (1) was used to isolate auxotrophic mutants, using D108 and Mu. Mutants were characterized by testing their ability to grow on ML minimal plates supplemented with glucose and various combinations of amino acids, nucleotides, and vitamins as described by Clowes and Hayes (6).

Phage-induced *thyA* mutants were isolated after formation of D108 or Mu lysogens (1) by plating on trimethoprim-containing minimal media as described by Miller (14).

Transductions. Transductions were performed using P1L4 and the methods described by Hull et al. (11).

RESULTS

D108 and Mu are heteroimmune. As D108 and Mu were found to be similar with respect to particle and plaque morphology and in their ability to act as generalized transducing phages (10, 15, 21), we sought to determine whether they share common immunity and host range properties. The ability of D108 and Mu to grow on various lysogenic bacterial strains is shown in Table 2. It can be seen that the plating efficiency of neither phage was inhibited by the presence of the other as a prophage. The success of D108 *cts10* phage development in a Mu lysogen was further examined in a one-step growth experiment. The results (Fig. 1) showed that the presence of Mu as a prophage had little or no effect on D108 phage development and further indicated that D108 and Mu were heteroimmune. The host range relationships among D108, P1, and Mu are also shown in Table 2. Cells resistant to D108 were resistant to P1 and vice versa (31

TABLE 2. *Host range and immunity relationships among bacteriophages D108, Mu, and P1*

Prophage or phage resistance[a]	Relative plating efficiency of:		
	D108	Mu	P1*kc*
None	1	1	1
D108 *cts10*	$<10^{-9}$	0.7	0.7
D108ʳ	$<10^{-9}$	10^{-9}	10^{-8}
Mu *cts62*	0.9	$<10^{-9}$	0.8
Muʳ	0.6	10^{-9}	0.2
P1*vir*ʳ	10^{-9}	$<10^{-9}$	10^{-7}

[a] All are derivatives of χ289.

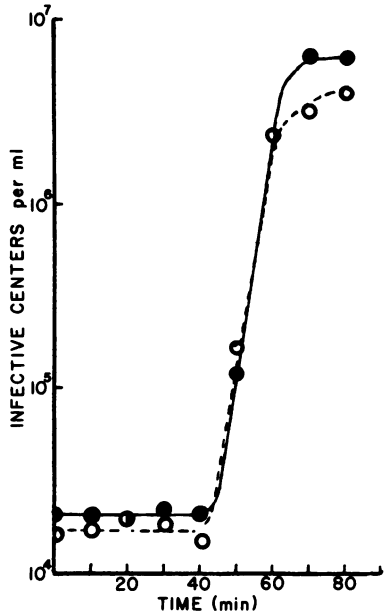

FIG. 1. *One-step growth kinetics of bacteriophage D108 on various E. coli derivatives. Experimental details are described in the text. Bacterial host strains are χ289 (●) and χ289 (Mu) (○).*

and 26 independent isolates tested, respectively). Although cells resistant to D108 were also resistant to Mu, the reverse was not necessarily true. Howe (10) has noted that most, but not all, P1-resistant strains are also resistant to Mu. Only a limited number (84) of D108-resistant χ289 derivatives were tested for resistance to Mu. The possibility that D108^r Mu^s derivatives existed at a low frequency, compared with D108^r Mu^r, could therefore not be excluded.

Mutation induction by D108. One of the most interesting properties of bacteriophage Mu is its ability to induce mutations in its bacterial host. To determine whether D108 would exhibit similar mutator properties, χ289 was infected with D108 by the method described by Boram and Abelson (1), and survivors were tested for the appearance of new auxotrophic markers. The frequency of auxotrophic mutants among survivors in D108-infected cultures was found to be 10- to 20-fold greater than in uninfected cultures (data not shown). Similar results were obtained with Mu. Mutations were identified in genetic loci specifying 19 different auxotrophic requirements. This suggests that D108, like Mu, is able to induce mutations at many different sites on the *E. coli* chromosome. D108-induced mutations in the *thyA* gene of χ289 were also selected, using trimethoprim to counterselect trimethoprim-sensitive *thyA*$^+$ cells. The frequency of *thyA* mutants in the infected culture was found to be 20- to 40-fold greater than that in the uninfected control (Table 3).

Properties of D108-induced *thyA* mutations. Several of the D108-induced *thyA* mutants were tested for their ability to revert to prototrophy. Mu-induced mutations do not revert under normal conditions (2). All eight of the D108-induced mutations tested were found to revert at a frequency of less than 10^{-10} (Table 3). To test for linkage between the D108-induced mutations and the D108 prophage, the *thyA* allele from each of two D108-induced mutants was transferred to χ1753 by P1L4-mediated cotransduction with *lysA*. Eight *thyA lysA*$^+$ recombinants from each transduction were purified and then tested for D108 immunity. All 16 were immune to D108. The frequency of D108 in the transducing lysates was 1,000-fold lower than that of P1, making double infection unlikely under the conditions used. Moreover, no D108-immune transductants were observed among 134 *his*$^+$ recombinants selected simultaneously. These findings support the notion that D108, like Mu, induces mutations by covalent insertion of its genome into regions of genetic function.

To further examine the degree of linkage, P1L4 phage grown on χ289 was used to transduce one of the *thyA lysA*$^+$ transductants described above to *thy*$^+$, and the purified recombinants were then tested for D108 immunity. The transductions were done with a multiplicity of infection of 1 for P1L4, and the infected cells were diluted 10-fold and spread on Davis-Mingioli minimal medium containing citrate; this procedure precluded infection of transductants with virulent P1L4 and prevented reinfection of potential D108$^-$ transductants with D108. All 86 *thyA*$^+$ transductants tested were found to have lost immunity to D108, suggesting that the site of D108 insertion is close to or identical with the site of the *thyA* mutation.

Prophage induction after lytic infection of a lysogen with heteroimmune phage. The

TABLE 3. *Mutation induction by bacteriophage*

Type of mutation	Frequency of:	
	thyA[a]	Reversion to *thyA*$^+$[b]
Spontaneous	8×10^{-7}	5×10^{-9}
Mu induced	2×10^{-5}	ND[c]
D108 induced	2×10^{-5}	$<2 \times 10^{-10}$

[a] For phage-infected cells, frequency was based on the survivors after infection.

[b] Reversion frequency shown of D108-induced *thyA* mutations was typical of eight mutants tested. The reversion frequency shown for spontaneous mutations was typical of four mutants.

[c] ND, Not determined.

following work was done to determine whether infection of a Mu lysogen by D108 *cts10* could result in increased recovery of the prophage type in the phage burst. Confluent plate lysates of D108 *cts10* were prepared at 37°C, using χ289 (Mu) as the host bacterium. The titer of Mu or D108 *cts10* in these lysates was determined by using χ289 (D108) or χ289 (Mu), respectively, as the indicator bacterium. The titer of Mu due solely to spontaneous induction was determined with a sham lysate which was prepared from a TYMC agar plate inoculated with χ289 (Mu) in soft agar without D108 *cts10*. The results are shown in Table 4. There was a 200-fold increase in the titer of Mu released from D108 *cts10*-infected cells as compared with the uninfected control. The reverse experiment, propagation of Mu *cts62* on a D108 lysogen, was also done, and a similar stimulation of D108 phage release was observed (Table 4).

The relative plating efficiency of the D108 *cts10*-Mu mixed lysates on χ289 Mu^r (D108) was also measured (Table 4) to determine the frequency of Mu-D108 genetic hybrids, since only phage with the immunity of Mu and host range of D108 ($i^{Mu}h^{D108}$) are able to form plaques on this host. These results show that about 4.5% of the phage with Mu immunity released after infection of the Mu lysogen with D108 *cts10* were Mu-D108 genetic hybrids with the host range of D108. Similarly, about 6% of D108 phage released upon infection of the D108 lysogen with Mu *cts62* acquired the immunity of Mu while retaining the D108 host range. For additional confirmation of the hybrid nature of phage forming plaques on χ289 Mu^r (D108), several plaques were picked, streaked individually for single-plaque isolates on χ289, and then tested for plaque-forming ability on several *E. coli* derivatives. Each isolate tested was able to form plaques on χ289, χ289 Mu^r, and χ289 (D108), but not on χ289 (Mu), and thus displayed the expected phenotype for $i^{Mu}h^{D108}$ phage. Although the reciprocal $i^{D108}h^{Mu}$ recombinant type could not be detected by direct plating because of the absence of a D108^r Mu^s (Mu) host, we have no reason to believe these recombinants did not exist. Therefore, we infer that about 10% of the prophage released upon superinfection with the heteroimmune phage acquired either the *i* or the *h* gene of the superinfecting phage.

The next question asked was whether the Mu phage genome released upon infection of a Mu lysogen with D108 *cts10* was often packaged in coats specifying the D108 host range. Thus, the titer of Mu in the D108 *cts10*-Mu mixed lysates was again determined, this time by preadsorbing the phage to χ289 Mu^r (D108) for 20 min at 37°C in L broth plus 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$, washing the infected cells free from unadsorbed phage, and then plating various dilutions to determine the titer of infective centers, with χ289 (D108) as the phage-sensitive indicator strain. Under these conditions, only $i^{Mu}h^{D108}$ recombinant phage and Mu prophage which had been released and then packaged into phage particles specifying the D108 host range could form infective centers, whereas all phage with Mu immunity, whether packaged with either Mu or D108 tails, could form plaques when plated directly on χ289 (D108). The results depicted in the first half of Table 5 suggested that the vast majority of Mu phage released from D108 *cts10*-infected Mu lysogens consisted of Mu DNA encapsidated in particles with D108 host range specificity.

To determine whether the increase in Mu prophage release is due to passive packaging of Mu DNA into particles with D108 host range or whether Mu gene expression is induced, it would be useful to assay for phenotypically mixed particles containing D108 DNA packaged in a Mu virion. This could be done by plating infective centers on χ289 (Mu) after preadsorption of phage to a D108^r Mu^s host. This experiment would reveal the number of particles with the prophage host range available for packaging phage DNA. However, as such a host strain is not currently available, this experiment was not done. Instead, the reverse experiment was performed; i.e., the titer of phage with Mu *cts62* immunity packaged in particles with D108 host range specificity was measured after infection of a D108 lysogen with Mu *cts62* by preadsorption of the lysate to χ289 Mu^r (D108) and subsequent removal of free phage. Under these plating con-

TABLE 4. *Titer of mixed phage lysates measured on various E. coli derivatives*

Infecting phage	Host prophage[a]	Phage titer (per ml) measured on:			
		χ289	χ289 (D108)	χ289 Mu^r, (D108)	χ289 (Mu)
D108 *cts10*	Mu	1×10^{10}	2×10^{7}	9×10^{5}	2×10^{10}
None	Mu	1×10^{5}	1×10^{5}	$<10^{2}$	$<10^{2}$
Mu *cts62*	D108	2×10^{9}	9×10^{8}	6×10^{5}	1×10^{7}
None	D108	1×10^{5}	$<10^{2}$	$<10^{2}$	1×10^{5}

[a] Infecting phage lysates were propagated on either χ289 (Mu) or χ289 (D108).

TABLE 5. *Titers of mixed phage lysates preadsorbed to χ289 Mu^r (D108)*

Lysate preparation (infecting phage:host)	Plating conditions[a]	Phage titer (per ml) measured on:	
		χ289 (D108)	χ289 Mu^r (D108)
D108 *cts10*: χ289 (Mu)	Direct	2×10^7	9×10^5
None:χ289 (Mu)	Direct	2×10^5	$<10^1$
D108 *cts10*: χ289 (Mu)	Preadsorbed	3×10^7	4×10^5
None:χ289 (Mu)	Preadsorbed	$<10^3$	$<10^3$
Mu *cts62*:χ289 (D108)	Direct	1×10^9	5×10^5
None:χ289 (D108)	Direct[b]	$<10^1$	$<10^1$
Mu *cts62*:χ289 (D108)	Preadsorbed	3×10^7	1×10^5
None:χ289 (D108)	Preadsorbed	$<10^2$	$<10^2$

[a] Phage were either diluted and mixed directly with cells in soft agar ("direct") or preadsorbed to χ289 Mu^r (D108) for 20 min at 37°C, washed twice to remove unadsorbed phage, and then diluted and plated in soft agar seeded with χ289 (D108) or χ289 Mu^r (D108) ("preadsorbed").

[b] Titer of this lysate measured on χ289 was 5×10^4/ml.

ditions, only phage that were genotypically $i^{Mu\ cts62}$ (with either h^{Mu} or h^{D108}) and which were packaged in particles with D108 host range specificity remained to form plaques on the χ289 (D108) host. The results of this experiment, depicted in the last half of Table 5 suggested that an increased number of phage particles with the D108 host range (3×10^7/ml) were available for packaging DNA after lytic infection of a D108 lysogen with Mu *cts62*, compared with the uninfected control D108 lysate (10^5/ml) (Table 4).

To investigate whether any of the above-described phenomena were due solely or partially to recombination between the superinfecting phage and the resident prophage, with synthesis of prophage-specified tail components only by recombinant phages, we repeated the superinfection experiments with *recA* hosts. Thus, D108 *cts10* was used to infect a *recA* Mu lysogen and Mu *cts62* was used to infect a *recA* D108 lysogen, with the resulting phage plated on the hosts used to collect the data for Tables 4 and 5 with and without preadsorption to χ289Mu^r(D108). Although the total yields of phage were reduced 10- to 100-fold, there was still a 100-fold increase in titer of phage with the prophage immunity, compared with the sham lysate, even though only about 0.1% of these phages were D108-Mu genetic hybrids. More importantly, phenotypic mixing did occur, and the same proportion of phage was packaged in particles with the host range specificity of the prophage as was observed in experiments with a *recA*$^+$ host (Table 5, last four lysate preparations).

DISCUSSION

The experiments described indicate that phage D108 is a mutator phage, like Mu, but is heteroimmune with Mu and has a different host range than Mu. In other experiments, we have found that the Mu and D108 genomes are 93% homologous, as revealed by heteroduplex and restriction enzyme analyses (G. S. Gill, R. A. Hull, and R. Curtiss III, manuscript in preparation). Like Mu, D108 appears to induce mutations by insertion of its genome into regions of genetic function on the host chromosome.

Infection of Mu lysogens by D108 *cts10* stimulates Mu phage release, and infection of D108 lysogens by Mu *cts62* stimulates D108 phage release. In both instances, about one phage with prophage immunity is released per 10 to 20 infected cells, and this is 100 times higher than the yield due to spontaneous prophage induction. Although a single-burst analysis is necessary to determine the number of phage with prophage immunity released per cell liberating such phage, the fact that almost 100% of the phage with Mu immunity released after infection of a Mu lysogen with D108 *cts10* were packaged in particles with D108 host range specificity is most compatible with the release of only one to several such phage per cell. Our experiments also reveal, at least in the case of Mu *cts62* infection of a D108 lysogen, that the number of superinfecting phage packaged in particles with the host range specificity of the prophage is about equal to the number of phage released with the prophage immunity genotype. Thus, in cells releasing phage with the prophage genotype, the prophage genes, at least for host range specificity, are expressed. To determine whether prophage release and/or expression of prophage genes was dependent on recombination between the superinfecting heteroimmune phage and the prophage, we repeated these experiments with *recA* hosts. Although the frequency of Mu-D108 genetic hybrids decreased from 10 to 0.1% of the phage with the immunity and/or host range of the prophage, the frequency of prophage release and the degree of phenotypic mixing leading to packaging of superinfecting phage in particles with prophage host range specificity and vice versa were identical to the values for infections with *recA*$^+$ hosts. Therefore, the prophage genome does not seem to be passively packaged under the direc-

tion of the superinfecting phage. The occurrence of prophage induction after superinfection with a heteroimmune phage in the D108-Mu family is in contrast to the near absence of such induction of P2 and λ prophages upon infection with immunity-insensitive mutants and/or heteroimmune phage (20). In addition, the recovery of prophage markers in these latter cases is most often associated with formation of recombinants with the superinfecting phage (20), a situation which occurs but apparently is not necessary for prophage induction in the Mu-D108 system. On the other hand, the recovery of λ with the density of prelabeled heavy bacterial DNA after infection of λ lysogens with λ i^{434} (17) implies that recombination might not be necessary for the rare prophage excision that occurs in the λ family.

In speculating on the mechanism of induced prophage release in the Mu-D108 system, two not mutually exclusive processes come to mind. In one model, the superinfecting phage would cause *trans*-activation of prophage genes to cause transcription of all or some prophage genes, including those that would lead either to overcoming repression and thus result in prophage release or directly to prophage release. Such *trans*-activation leading to expression of λ prophage late genes does occur after superinfection with a heteroimmune phage, although such *trans*-activation does not lead to expression of positive control genes that can overcome immunity repression (19). An alternative model is based on the need for Mu, and presumably D108, to integrate into the host chromosome as part of the normal lytic cycle (2, 3, 16) and the existence of extensive homology between the Mu and D108 genomes (Gill et al., manuscript in preparation). It is thus possible that the infecting heteroimmune phage sometimes integrates at a site of homology next to or in the resident prophage, to result in replication intermediates that are Mu-D108 concatenations. The distinction between these and other possibilities will necessitate use of Mu and D108 mutants, isolation and characterization of Mu and D108 gene products, and isolation and characterization of replication intermediates.

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