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Bacteriophage P1 as a Vehicle for Mu Mutagenesis of *Salmonella typhimurium*

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We developed a procedure using bacteriophage P1 as a vector for transferring Mu phage deoxyribonucleic acid into *Salmonella typhimurium*. Mu phage transferred in this manner yielded lysogenic auxotrophs, and we demonstrated that specific deletions and *lac* gene fusions can be selected.

The bacteriophage Mu has been an important asset to genetic studies of *Escherichia coli* because of its mutagenic properties. Insertions of Mu phage DNA into the host chromosome occur at a large number of sites and may be used to promote deletions, inversions, transpositions, and gene fusions (16).

We have been studying the regulation of nitrogen utilization by *Salmonella typhimurium* and were interested in exploiting the mutagenic capabilities of Mu phage. However, wild-type *S. typhimurium* is not sensitive to infection by Mu. Although Mu-sensitive mutants could have been selected in a manner similar to that used for *Klebsiella pneumoniae* (1), we wanted a procedure that would allow the use of Mu with a variety of existing *S. typhimurium* strains. Our approach was to use phage P1 as a vehicle for transferring Mu DNA into P1-sensitive *S. typhimurium* strains. P1 has more than twice the DNA packaging capacity of Mu and can transduce Mu insertion mutations (4, 9). Thus, we considered using P1 specifically as a vector for Mu mutagenesis of *S. typhimurium*. We have demonstrated that not only can P1 serve as a vector for Mu, but that this procedure can be used for obtaining auxotrophs, deletion mutations, and *lac* fusions in *S. typhimurium*.

The Mu phage used in this investigation, Mu *cts* d1(Ap^r *lac*) was constructed and characterized by M. Casadaban (5; obtained from G. Stauffer). This defective phage generates random mutations in *E. coli* like wild-type Mu, but it has several advantages. The Mu *cts* d1(Ap^r *lac*) phage has a temperature-sensitive repressor protein which facilitates the induction of lysogens. It also contains a gene conferring ampicillin resistance (Ap) derived from a Tn*3* transposon that allows the direct selection of ampicillin-resistant Mu lysogens. The Mu *cts* d1(Ap^r *lac*) has the *lac* structural genes but is devoid of the *lac* promoter and operator. Thus, insertion of this phage in the correct orientation allows a single-step fusion of the *lac* genes to foreign promoters.

The following procedure was used initially to prepare a P1 lysate capable of transducing Mu into *S. typhimurium* strains. An *E. coli* Mu lysogen, strain MAL103 (5; Table 1), was grown to saturation at 30°C in Luria broth (LB) medium (3) containing 10 mM $CaCl_2$. A lysate of P1CM *clr*100 phage (15) was cross-streaked against the MAL103 cells on LB plates containing 10 mM $CaCl_2$ and chloramphenicol (12.5 μg/ml). The plates were incubated at 30°C overnight, and chloramphenicol-resistant cells arising at the phage-cell intersection were picked and purified on LB plates containing ampicillin (30 μg/ml) and chloramphenicol (12.5 μg/ml). This strain was inoculated into 50 ml of super broth (8) containing $MgSO_4$ (2 mM). The culture was grown with aeration at 32°C until an optical density of 20 Klett units (2×10^8 cells/ml) as measured on a Klett Summerson colorimeter (red filter no. 62) was reached. The culture was centrifuged at 8,800 × *g* for 10 min and resuspended in 1/10 the original volume of fresh super broth preheated to 42°C and containing 2 mM $MgSO_4$. The cells were incubated with aeration for 20 min at 42°C for phage induction. The culture was then shifted to 37°C and incubated for 2 h to allow lysis. A 0.2-ml portion of chloroform was added to the culture, and after 5 min the cell debris was removed by centrifugation at 3,400 × *g* for 15 min. The supernatant was stored with chloroform. This preparation contained about 3×10^9 plaque-forming units (PFU) per ml consisting of both P1 and Mu phage. The concentration of P1 phage alone was determined by infecting a Mu phage-resistant strain of *E. coli* and was about 6×10^8 PFU/ml.

The transduction of Mu *cts* d1(Ap^r *lac*) by the P1 phage lysate was measured by selection for transductants having an ampicillin-resistant phenotype. The *S. typhimurium* strain, JB1396 (Table 1), was used as the recipient in these transductional crosses. The *galE* mutation in this strain is necessary to allow P1 infection, and the mutations affecting DNA restriction decrease degradation of *E. coli*-derived DNA in

TABLE 1. *Bacterial strains*

Strain[a]		Genotype/phenotype
MAL103		F⁻, Mu *cts* d1(Ap^r *lac*), Mu *cts*, Δ(*proAB lacIPOZYA*)*XIII strA*
CB109	Derived from	MAL103 as a (P1CM *clr*100) lysogen
JB1396		*metA22 metE551 trpB2 xyl-404 galE496 hspLT6 hspS29 ilv-452 strA120* Hl-b *nml H2-e, n, x* (Fels2)⁻ *flaA66*
JB1401	Derived from	JB1396 as *glt*::Mu *cts* d1(Ap^r *lac*)
JB1408	Derived from	JB1396 as *glt*::Mu *cts* d1(Ap^r *lac*) P1CM *clr*100
JB1462		*gal glpR1^c glpD4 malA asm-103*
JB1477	Derived from	JB1462 as *gln*::Mu *cts* d1(Ap^r *lac*)
JB1636	Derived from	JB1462 as Mu *cts* d1(Ap^r *lac*)

[a] All strains are *S. typhimurium* except MAL103 and CB109, which are *E. coli*.

the P1 particles (7, 17). Strain JB1396 was grown to saturation at 30°C in LB medium containing 10 mM $CaCl_2$. A 0.1-ml portion of the phage preparation grown on MAL103 was added to a 0.1-ml portion of JB1396 cells (a multiplicity of 0.3 P1 PFU/cell). The mixture was incubated for 1 h at 30°C without shaking. A 2.0-ml portion of LB was added, and the culture was incubated an additional hour with shaking to allow expression of ampicillin resistance. The cells were centrifuged at 12,000 × *g* for 10 min and washed twice with 0.85% NaCl to remove exogenous β-lactamase from the media. The cells were resuspended in 2.0 ml of 0.85% NaCl, a 0.2-ml portion was plated onto LB agar containing 30 μg of ampicillin per ml, and the plates were incubated at 30°C for 16 to 24 h. Controls demonstrated that the plating of either cells or phage lysate alone did not yield ampicillin-resistant colonies. Furthermore, a similar procedure with a Mu lysate did not produce any ampicillin-resistant colonies, showing that the transduction was not due to the Mu phage.

The selection plate had ca. 400 ampicillin-resistant transductants of which 1.0% (3/276) were auxotrophs. These auxotrophs were purified and shown to require glutamate, histidine, and adenine. These results suggested that the Mu *cts* d1(Ap^r *lac*) phage could be transduced via P1 and prompted us to perform a large-scale experiment to quantitate the frequency and distribution of auxotrophs obtained with this technique.

To make this procedure amenable to the majority of *S. typhimurium* strains which unlike JB1396 possess functional DNA restriction enzymes, we prepared a P1CM *clr*100 lysate on the glutamate auxotroph, JB1401 (Table 1), obtained in the initial selection for Mu *cts* d1(Ap^r *lac*) lysogens. The preparation of the P1 lysate and the transduction procedure were performed exactly as described above except JB1401 was the donor and JB1462 (Table 1) was the recipient. Approximately 20,000 ampicillin-resistant transductants were obtained, of which 4,000 were picked and scored for auxotrophy. About 1.5% of the ampicillin-resistant transductants were auxotrophs (Table 2). The high frequency of certain auxotrophs (i.e., cysteine and histidine) may reflect the large number of genes involved in the synthesis of these amino acids. Alternatively, these auxotrophs may represent siblings since the growth needed for expression of ampicillin resistance does not insure that each mutant is independent. The above results show that Mu can be an effective mutagen in *S. typhimurium* and a large variety of mutants can be obtained as in *E. coli*. It is also clear that the selection for ampicillin resistance is giving rise to Mu insertions at locations other than that in the donor strain JB1401. This result was expected since our procedure did not restrict Mu DNA replication during induction of CB109 nor upon infection of JB1396.

We also wanted to determine whether the Mu *cts* d1(Ap^r *lac*) derivative would be useful for constructing *lac* operon fusions in *S. typhimurium* analogous to those in *E. coli* (5). Such fusions are extremely useful for studying regulation of gene activity. Since *S. typhimurium* does not have *lac* genes, β-galactosidase expression in the ampicillin-resistant transductants is dependent upon the Mu *cts* d1(Ap^r *lac*) being fused to a foreign promoter. Among 156 ampicillin-resistant transductants tested, 45 (30%) expressed β-galactosidase to various extents on glucose media containing ampicillin (30 μg per ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (12). Presumably the other 70% of the ampicillin-resistant transductants which do not express β-galactosidase have Mu *cts* d1(Ap^r *lac*) inserted in the opposite orientation or have the phage inserted in genes not expressed under these growth conditions.

One glutamine auxotroph, JB1477 (Table 1), isolated during the screening of ampicillin-resistant transductants clearly produced β-galactosidase on glutamine-supplemented indicator agar and was characterized further to demonstrate that the glutamine requirement and *lac*

TABLE 2. *Classes of auxotrophic mutants isolated by P1 Transduction of Mu cts d1(Ap^r lac)*

Supplement required for growth	No. of auxotrophs[a]
L-Cysteine	17
L-Histidine	8
L-Methionine	8
Uracil	3
Adenine	2
L-Glutamate	2
L-Proline	2
L-Serine or L-glycine	2
L-Isoleucine, L-leucine, and L-valine	1
L-Leucine	1
L-Threonine	1
L-Glutamine	1
Unknown	9

[a] Ampicillin-resistant transductants were selected as described in the text, and 4,000 were scored for their ability to grow on a glucose-minimal salts medium. Mutants unable to grow on minimal medium, but able to grow on supplemented medium (LB), were purified and their requirement was determined.

expression were caused by a single Mu insertion. The mutation causing the glutamine auxotrophy was in the *glnA* region, the structural gene for glutamine synthetase, since F' 133 (2) could complement the *gln* mutation. The glutamine auxotroph could be transduced to glutamine prototrophy with P22 HT105/1*int* phage (13) grown on a strain having Tn*10* linked to the *glnA* region (14). About 5.0% of the Tet^r transductants were *gln*$^+$, sensitive to ampicillin, and did not produce β-galactosidase. Thus, the *gln* mutation is due to an insertion of a single Mu *cts* d1(Ap^r *lac*) phage within or near the structural gene for glutamine synthetase.

To determine whether the *lac* genes were now fused to the promoter for glutamine synthetase, we measured the β-galactosidase activities using cells grown in media that influence glutamine synthetase regulation. The results show that β-galactosidase is regulated similarly to glutamine synthetase in a control strain, JB1636, suggesting that the Mu *cts* d1(Ap^r *lac*) may be inserted in the *glnA* gene (Table 3), although β-galactosidase levels in JB1477 do not increase to the same extent as glutamine synthetase in JB1636 during growth in glucose-glutamate medium. Differences in derepression ratios have been observed with various *lac* fusions in other genes and may be due to the creation of additional control signals (5, 11).

E. coli deletion mutants have been selected based upon survival of temperature-sensitive Mu lysogens at elevated temperatures (8, 10). We demonstrated that this procedure works with the Mu *cts* d1(Ap^r *lac*) in *S. typhimurium* by selecting heat-resistant survivors of JB1477 at 43°C. The majority of cells are killed at this temperature due to the thermoinducibility of Mu *cts* d1(Ap^r *lac*), but cells which survive this heat treatment and are ampicillin sensitive contain deletions extending within and beyond the *glnA* gene (data not shown).

In addition to the scoring of auxotrophs (Table 2), we selected specific mutants by scraping the ampicillin-resistant transductants from the selective plates and preparing a pooled cell culture. This culture was subjected to cycloserine counter selection followed by enrichment for the desired auxotroph. This technique has been used to successfully to isolate glutamate dehydrogenase mutants, some of which have Mu *cts* d1(Ap^r *lac*) oriented such that β-galactosidase is under the glutamate dehydrogenase promoter control.

The experiments presented in this paper have shown that Mu phage in *S. typhimurium* is able to promote chromosomal rearrangements similar to those observed for *E. coli*. In fact, selection for Mu *cts* d1(Ap^r *lac*) fusions is more easily accomplished in *S. typhimurium* because of the absence of *lac* genes; similar selections in *E. coli* require construction of derivatives with the *lac* genes deleted. An additional advantage of our procedure is that the P1 lysates are considerably more stable and can be stored longer times than Mu lysates; thus, this procedure may be valuable for general use even with Mu-sensitive strains. Another advantage to our system is its potential for use in any other bacterial strains having a P1 host range. For example, P1-mediated transduction of Mu lysogens between genera could be

TABLE 3. *Regulation of enzyme levels in a strain with a gln::Mu cts d1(Ap^r lac) insertion*

Strain	Growth medium[a]	Enzyme activity	
		Glutamine[b] synthetase	β-Galactosidase[c]
JB1636[d]	Glucose-ammonia	96	0.20
	Glucose-glutamate	512	0.25
JB1477	Glucose-ammonia	0	19
	Glucose-glutamate	0	43

[a] Glucose-ammonia and glucose-glutamate are minimal salts media (3) containing glucose (0.4%) as the carbon source and $(NH_4)_2SO_4$ (17 mM) or L-glutamate (20 mM) as the nitrogen source, respectively. All media include L-glutamine at 2.5 mM.

[b] The assay is the γ-glutamyl transferase reaction (6) and specific activity is expressed as nanomoles of γ-glutamyl hydroxymate formed per minute per milligram of protein.

[c] The assay was performed by the method of Miller (12) by using sonicated cell extracts. Specific activity is expressed as micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

[d] Strain JB1636 is a prototroph with a Mu *cts* d1(Ap^r *lac*) insertion at an unknown location and is used as a Gln$^+$ isogenic control for these experiments.

useful for probing differences among bacterial strains and for developing genetic systems in new strains without having to select Mu-sensitive derivatives.

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