Behaviour of Temperate Phage Mu in *Salmonella typhi*

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We have developed a convenient system for genetic analysis of *Salmonella typhi* exploiting the properties of the mutator phage Mu. In spite of the fact that wild-type *Salmonella typhi* strains do not allow Mu to form plaques on them, we have shown that these strains are actually sensitive to the phage. It proved possible to use Mu to induce mutations and to promote intra- and interspecific genetic transfer, without having to introduce the phage into the bacteria by means other than infection. Furthermore, we isolated *Salmonella typhi* derivatives on which Mu formed plaques, and studied the behaviour of Mu in these and wild-type strains.

INTRODUCTION

The Gram-negative enterobacterium *Salmonella typhi* is the causative agent of typhoid fever in humans. Different biological aspects of this bacterium have been investigated since its discovery; however, the genetics of the organism have not been systematically studied because a convenient system to manipulate its chromosome has not been developed. Phages are currently useful tools for the genetic study of bacteria. Among them, Mu appears particularly interesting, being a temperate phage that can insert its DNA randomly into the host genome and induce polar mutations at high frequency which are very stable and not leaky (Taylor, 1963; Martuscelli *et al.*, 1971). Like other transposons, Mu induces various genetic rearrangements such as deletions, inversions, replicon fusions and transpositions of host DNA fragments (for review see Toussaint & Résibois, 1983). Furthermore, Mu is a generalized transducing phage, even though the efficiency of transduction is not as good as that promoted by phage P1 (Howe, 1973; Howe & Bade, 1975).

Mu host range is determined by the products of at least two pairs of phage genes, S and U, S' and U', one set being silent while the other is expressed. Which pair is expressed depends on the orientation of the invertible G region of the phage genome (Van de Putte *et al.*, 1980). Two phage types G(+) and G(–), recognize different cell surface receptors, which in both cases are located in the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria (Kamp & Sandulache, 1983). Some bacteria, such as *Escherichia coli* K12 and '*Arizona hinshawii*' are sensitive to Mu particles having the specificity corresponding to the G region in the + orientation, while others, such as *Citrobacter freundii* and *Erwinia chrysanthemi* are sensitive to phage particles having the specificity corresponding to the opposite (–) orientation of the G region (Van de Putte *et al.*, 1980). Most bacterial species, including *Salmonella typhimurium*, are naturally resistant to phage Mu. However, Faalen *et al.* (1981) fortuitously found a *S. typhimurium* mutant on which Mu plaques. The mutation leading to sensitivity to Mu was shown to be linked to the *his* operon. Moreover, *S. typhimurium* strains with large deletions in the

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Table 1. *Bacterial strains and phages*

Strains	Characters	Source or reference
<i>E. coli</i>		
C600	Leu ⁻ Thr ⁻ Thi ⁻ Gal ⁻ <i>lacY tonA supE</i>	Appleyard (1954)
C600(Mucts62)	As C600, lysogen with Mucts62	This work
MXR(Mucts62pAp1)	Δ (<i>pro-lac</i>) <i>galE</i> Thi ⁻ <i>recA53</i> , lysogen for Mucts62pAp1	Faelen <i>et al.</i> (1978)
MXR(Mucts62pAp1)/F' Lac ⁻ Pro ⁻ (Mul8A1)	As MXR(Mucts62pAp1) with F' Lac ⁻ Pro ⁻ with Mul8A1	Faelen <i>et al.</i> (1979)
HB94	HfrH Hsr ⁻ Gal ⁻ (λ c ⁺)	G. Alfaro, UNAM, Mexico
<i>S. typhimurium</i>		
MA766	HfrK9 <i>thr-49 hisG9424::Tn10</i> Δ 4 Δ 11 <i>musA1</i>	Faelen <i>et al.</i> (1981)
ZJ1001	Thr ⁺ revertant of MA766	This work
<i>S. typhi</i>		
JM1382	<i>cym</i> ^{ts} (requirement for cysteine or methionine) Lac ⁻ Nal ^R	Martuscelli <i>et al.</i> (1971)
ZJ1	JM1382 <i>hisG9424::Tn10</i> Δ 4 Δ 11 <i>musA1</i>	This work
ZJ2	His ⁺ spontaneous revertant of ZJ1	This work
ZJ11	His ⁺ spontaneous revertant of JM1382 <i>hisG9424::Tn10</i> Δ 4 Δ 11 (Mup ⁺)	This work
MS22	ZJ2(Mucts62pAp1) Ura ⁻	This work
Phages		
Mucts62	Thermoinducible	Howe (1973)
Mucts62pAp1	Tn3 in the G region	Leach & Symonds (1979)
Mul8A1	Mucts62pAp1 Δ (B-S)	Lefebvre & Toussaint (1981)
Mucts62 <i>mom3452</i>	Mucts62 modification-deficient	Toussaint (1976)

his region are also able to support the growth of Mu. From this observation and similar ones obtained with *Klebsiella pneumoniae* (Bachhuber *et al.*, 1976) it has been proposed that sensitivity to Mu is due to the loss of a bacterial function.

It has also been observed that Mu can develop in a large number of hosts on which the phage can not adsorb, provided it is introduced into the cell by other means, such as on a broad host-range conjugative plasmid like RP4 (Murooka *et al.*, 1981). In this paper we report the use of phage Mu as a genetic tool to study *S. typhi*.

METHODS

Bacteria and phages. These are listed in Table 1.

Media. Bacteria and phages were grown in L broth (Miller, 1972). Mu lysates were titrated in L agar. Fry medium (Fry, 1959) was used to determine the sensitivity of the strains to phage Mu and to isolate Mu-bacterial lysogens. Minimal medium was M9 (Miller, 1972) with 0.2% (w/v) glucose. When required, amino acids were added at a concentration of 40 μ g ml⁻¹ with the exception of glutamine, serine and proline, which were added at 150 μ g ml⁻¹. For growth of *S. typhi*, Na₂S₂O₃ was added to M9 at a concentration of 100 μ g ml⁻¹. The Lac⁻ phenotype was tested on MacConkey agar supplemented with 1% (w/v) lactose.

Antibiotics were used at a concentration of 25 μ g ml⁻¹ for ampicillin (Amp) and nalidixic acid (Nal) and 100 μ g ml⁻¹ for tetracycline (Tet). Phages were diluted in SM buffer (0.01 M-Tris/HCl, 0.1 M-NaCl, 0.02 M-MgSO₄, pH 7.4) and bacteria in 0.02 M-MgSO₄.

Preparation of phage lysates. Phage lysates were prepared by heat induction of lysogens following the procedure described by Bukhari & Ljungquist (1977).

Mu adsorption. Mu lysates were prepared by induction of a lysogen and the non-adsorbed phages were measured. Phages and bacteria were mixed at an m.o.i. of 3 for 15 min at 37 °C and then the bacteria removed together with adsorbed phages by centrifugation. Phages remaining in the supernate were titrated.

Lysogenization by Mu. A lawn of exponentially grown bacteria was spread on an L agar plate, and a drop of concentrated lysate of Mucts62pAp1 was spotted onto the lawn and incubated overnight at 32 °C. Surviving bacteria isolated from the lysis area were then tested for Amp^R and the presence of Mucts62 (i.e. immunity at 32 °C, and lysis and phage production at 42 °C).

Infection experiments. The procedure described by Bukhari & Ljungquist (1977) was used.

Transduction experiments. These were done as described by Howe (1973).

Matings. These were done following the procedure of Miller (1972).

RESULTS AND DISCUSSION

Sensitivity of S. typhi to Mu

Our laboratory possesses an extensive collection of *S. typhi* strains isolated from patients of the 'La Raza' hospital, Mexico City. These strains are classified with respect to the surface Vi antigen (Anderson & Williams, 1956). Many such clinical isolates of different Vi types were tested with Mu lysates prepared after induction of a lysogen. Such lysates contain a mixture of approximately 50% of particles with the host specificity corresponding to the G region in the + orientation and approximately 50% of particles with the host specificity corresponding to the opposite orientation of G (Van de Putte *et al.*, 1980). None of the *S. typhi* strains tested allowed Mu to form plaques under the conditions described for Mu growth in *E. coli* K12 (Bukhari & Ljungquist, 1977). However, Amp^R bacteria were isolated after infection with Mu_{cts62pAp1}, a Mu derivative which has part of its G region substituted by a portion of Tn3 (Leach & Symonds, 1979). The analysis of these Amp^R bacteria showed that they all contained a complete Mu_{cts62pAp1} genome, since they produced infective particles after thermal induction. We concluded that Mu can infect *S. typhi* in spite of its inability to form plaques on this organism, and that *S. typhi* is sensitive to Mu particles with the specificity determined by the G region in the + orientation, because the phage used has the G loop blocked in this orientation.

Following this observation we decided to isolate *S. typhi* strains on which Mu plaques (i.e. Mup⁻ strains) and study the development of Mu in wild-type strains (Mup⁺).

Isolation of Mup⁻ strains

S. typhimurium strains are naturally resistant to Mu infection, but Faalen *et al.* (1981) found a mutant on which Mu plaques. As the *musA1* mutation which leads to sensitivity is linked to the *his* operon, these authors labelled the *hisG* gene of the sensitive strain with Tn10Δ4Δ11, which can excise but not transpose. The *musA1* and *hisG* :: Tn10Δ4Δ11 markers were introduced into an HfrK9 *S. typhimurium* so that both markers were transferred early by the donor strain during conjugation (Faalen *et al.*, 1981).

S. typhi and *S. typhimurium* are closely related species and hybrid derivatives have been isolated (Diena *et al.*, 1973). It was thus reasonable to expect to isolate Mup⁻ strains of *S. typhi* by introducing the *musA1* character of *S. typhimurium*. In fact, previous experiments (L. Topisirovic, personal communication) showed that the *musA1* marker transferred into some Yugoslavian *S. typhi* strains was sufficient to allow Mu to form plaques on them. Therefore, we mated Nal^R derivatives of *S. typhi* with MA766 for 30 min and selected the Tet^R Nal^R colonies. These were obtained with frequencies ranging from 1.5×10^{-6} to 3.5×10^{-6} . The analysis of these recombinants showed that all of them had the His⁻ phenotype of the donor strain and that 70% allowed Mu to form plaques. The Mup⁻ derivatives retained the Vi type of their *S. typhi* parent.

Behaviour of Mu in Mup⁻ and Mup⁺ strains

The ability of the different strains to adsorb phage Mu was determined as described in Methods. We found that Mup⁺ and Mup⁻ strains adsorbed phage Mu with the same efficiencies (see Table 2). This implied that the absence of phage plaques was not due to an insufficient adsorption of Mu on *S. typhi* Mup⁺ membranes.

To measure the level of lysogenization, *S. typhi* was infected with the phage Mu_{cts62pAp1} as described in Methods. We observed that 40% of the survivors of Mup⁻ strains were lysogens, the actual frequency of lysogenization being 4.5×10^{-4} . It should be noted that the lysogenization frequency in the wild-type strains (Mup⁺) was 4×10^{-5} , i.e. 10-fold lower (Table 3). However, both Mup⁺ and Mup⁻ Mu_{cts62pAp1} lysogens gave a burst of about 10^9 infectious particles upon heat induction. This showed that the transposition level was equal in these strains, as transposition is the mechanism required for Mu replication during lytic growth.

As the efficiency of adsorption and the transposition frequency were the same in Mup⁺ and Mup⁻ strains, we determined whether the efficiency of Mu DNA injection was a limiting factor in Mup⁺ as compared to Mup⁻ strains. An experiment was designed that would enable distinction between phages actually propagated inside *S. typhi* and those that were carried by the

Table 2. *Effect of Mucts62 mom3452 development upon infection of S. typhi Mup⁺ and Mup⁻ strains*

Representative results of at least three determinations; variation in the results was 5 to 10%.

	JM1382 (Mup ⁺)	ZJ2 (Mup ⁻)
No. of bacteria per ml	4.4×10^8	3.6×10^8
Phage particles per ml	9.0×10^8	9.0×10^8
Unadsorbed phages per ml	2.1×10^8	2.1×10^8
Bacteria surviving infection	2.2×10^8	4.0×10^7
Infective centres titrated on:		
<i>E. coli</i> r ⁻ m ⁺	7.3×10^7	2.8×10^8
<i>E. coli</i> r ⁺ m ⁺	1.4×10^7	5.0×10^7
Phage burst, 80 min after infection, titrated on:		
<i>E. coli</i> r ⁻ m ⁺	1.2×10^9	4.0×10^9
<i>E. coli</i> r ⁺ m ⁺	1.5×10^7	1.0×10^8

Table 3. *Lysogenization of S. typhi Mup⁺ and Mup⁻ strains by Mucts62pAp1*

Representative results of at least three determinations; variation in the results was 5 to 10%.

	Infection by Mucts62pAp1 of	
	JM1382 (Mup ⁺)	ZJ2 (Mup ⁻)
Frequency of lysogenization	4.0×10^{-5}	4.5×10^{-4}
Percentage of auxotrophs induced	3	3
Phage burst per ml after thermal induction of lysogens	1.0×10^9	9.5×10^8

bacteria as non-adsorbed phages not removed by centrifugation. The fact that *S. typhi* does not possess an effective system to restrict DNA with *E. coli* K12- or *S. typhimurium*-specific modifications was exploited. This has been inferred from its ability to receive plasmids, such as F and resistance plasmids, during interspecific matings (G. Alfaro, personal communication), and the observation that the e.o.p. of phages Mu propagated in *E. coli* K12 or *S. typhimurium* is the same on *S. typhi* Mup⁻ strains as on the original strain (data not shown). Furthermore, Mucts62 *mom3452* (Toussaint, 1976), a phage deficient in its own DNA modification system and that is consequently more sensitive to host restriction, was used.

A Mucts62 *mom3452* lysate was prepared by induction of an *E. coli* K12 lysogen and used to infect *S. typhi* Mup⁺ and Mup⁻ strains in parallel. Phages that actually infected *S. typhi* and propagated in it should no longer have the *E. coli*-specific DNA modification, while phages carried should retain it. Therefore, infective centres and phages produced upon infection were titrated on *E. coli* K12 restriction + and restriction - strains. The difference between the two bursts gives the number of phages that have lost *E. coli* K12 modification. The results (Table 2) show that the number of *S. typhi* infective centres was slightly but reproducibly lower (three- to four-fold) in the wild-type with respect to the Mup⁻ strain. This difference was maintained at the level of phage bursts produced upon infection and at the level of bacteria surviving infection. This suggests that DNA injection is less effective in Mup⁺ than in Mup⁻ strains and could be an explanation for the absence of visible plaques in Mup⁺ strains. Nevertheless, this defect is not necessarily the only one, since the difference in the efficiencies of lysogenization between the Mup⁺ and Mup⁻ strains (10-fold) seems too large to be completely explained by the difference in DNA injection efficiency.

Isolation of auxotrophic strains

One of the most distinctive features of Mu is its mutator property, which results from its random insertion into the host genome (Taylor, 1963, Martuscelli *et al.*, 1971). This property was used to isolate auxotrophs of *S. typhi*. For this purpose, the Mup⁻ strain ZJ2 was lysogenized with Mucts62 or Mucts62pAp1. The percentage of auxotrophs obtained (2 to 3% of lysogens)

Table 4. Transduction between strains of *S. typhi* and between *S. typhi* and *E. coli*

Representative results of at least three determinations; variation in the results was 5 to 10%. In both intraspecific and interspecific transduction the m.o.i. was 0.5.

	Donor	Recipient	Selective marker	Total transduction frequency	Percentage mini-Mu-duction
<i>Intraspecific transduction</i>					
1.	Mup ⁻	Mup ⁻			
	ZJ1(Mucts62)/F' Lac ⁻ Pro ⁻ (Mu18A-1)[<i>hisG</i> ::Tn10Δ4Δ11]	ZJ2 [His ⁺]	Tc ^R	9.0 × 10 ⁻⁶	14
	ZJ2(Mucts62)/F' Lac ⁻ Pro ⁻ (Mu18A-1)[His ⁺]	ZJ1 [<i>hisG</i> ::Tn10Δ4Δ11]	His ⁺	8.5 × 10 ⁻⁶	—
2.	Mup ⁺	Mup ⁻			
	JM1382 (Mucts62pAp1)/F' Lac ⁻ Pro ⁻ (Mu18A-1) [Ura ⁺]	MS22 (Mucts62pAp1) [His ⁺ Ura ⁻]	Ura ⁺	1.0 × 10 ⁻⁵	—
3.	Mup ⁻	Mup ⁺			
	ZJ1(Mucts62)/F' Lac ⁻ Pro ⁻ (Mu18A-1)[<i>hisG</i> ::Tn10Δ4Δ11]	ZJ11 [His ⁺]	Tc ^R	1.1 × 10 ⁻⁶	14
<i>Interspecific transduction</i>					
	<i>S. typhi</i>	<i>E. coli</i>			
	ZJ1(Mucts62)/F' Lac ⁻ Pro ⁻ (Mu18A-1)	C600	Tc ^R	3.7 × 10 ⁻⁷	90

was the same as that observed in *E. coli* K12 (Howe, 1973); 26 of the auxotrophs were characterized and 11 different requirements were identified, suggesting that Mu can also insert into a large number of sites in *S. typhi*. The reversion frequency of these mutations was lower than 10⁻¹⁰, with the exception of two mutations for which reversion was around 10⁻⁸. For one of the mutants (MS22, Ura⁻), transduction of the wild-type allele resulted in the loss of the Mu prophage, showing that a Mu genome was closely linked to the mutated gene.

Transduction

Mu mediates generalized transduction at low frequency (Howe & Bade, 1975). However, this frequency is 10- to 100-fold higher when mini-Mu/Mucts62 mixed lysates are used (Faellen *et al.*, 1978, 1979). A mini-Mu is a phage with a large internal deletion and lacks the lytic and most of the morphogenetic functions. It can insert and transpose but is unable to accomplish a lytic cycle. However, in the presence of a helper phage such as Mucts62, the mini-Mu can be encapsidated with a piece of host DNA at one of its extremities. This is probably the reason for its increased transducing capacity. Moreover, a particular mechanism of transduction called mini-Mu-duction can also occur. Indeed, bacterial DNA flanked by two mini-Mu in the same orientation can be encapsidated by the helper phage. Upon infection of a sensitive bacterium, the mini-Mu/host DNA/mini-Mu sequence can insert at random into the bacterial chromosome using Mu transposase. The mini-Mu we used, Mu18A-1, carries the ampicillin resistance marker of Mucts62pAp1. Strains carrying Mu18A-1 and Mucts62 were constructed by introducing an F' Lac⁻ Pro⁻ (Mu18A-1) episome into various *S. typhi* strains containing the Mucts62 prophage. The dilysogens were heat-induced and the mixed lysates obtained were used to transfer genetic material between different *S. typhi* strains as follows: (1) from Mup⁻ to Mup⁻ strains; (2) from Mup⁺ to Mup⁻ strains; (3) from Mup⁻ to Mup⁺ strains (Table 4).

In the three situations analysed, the highest frequency of transduction was obtained at low m.o.i (0.5 to 1) (data not shown). When the recipient was a Mup⁻ strain, whatever the mixed lysate used (1 and 2, Table 4), the transduction frequency was quite high (~10⁻⁵) and comparable to that observed in *E. coli* K12 with the same system (Faellen *et al.*, 1979). Consequently, there is a good probability of transducing any genetic marker of the *S. typhi* genome. Moreover, Tet^R transductants were found that maintained the His⁺ character of the recipient and acquired both Mu immunity and the ampicillin resistance marker (1, Table 4). These transductants were probably the result of mini-Mu-duction, since ampicillin resistance is a marker of Mu18A-1; these lysogens survive heat induction and do not produce phage particles.

Furthermore the acquisition of Tet^R without loss of the His⁻ phenotype means that there was no homologous recombination. In the case of genetic transfer from Mup⁺ to Mup⁻ strains (2, Table 4) it was not possible to test mini-Muduction because the recipient was a lysogenic strain. When the recipient was wild-type (3, Table 4), the frequency of transduction was lower. This can be related to a similar reduction (sevenfold) of the lysogenization frequency observed in these strains.

Mini-Muduction allows insertion of DNA without sequence homology. We tried to exploit this property for isolation of *S. typhi*-*E. coli* K12 hybrids, by transferring the *hisG*:Tn10Δ4Δ11 region from ZJ1 into C600. Experimental conditions were the same as for intraspecific transduction. The frequency of transduction was low but still reasonable (Table 4), given that the *E. coli* K12 strain used as recipient possessed an effective DNA restriction system. Almost all the transductants (90%) seemed to be mini-Muductants, since in addition to the selected marker, Tet^R, they also received Amp^R and Mu immunity; moreover, they retained the His⁺ allele of the recipient. Nevertheless, some true transductants were found. This can be explained by the partial homology, observed by DNA-DNA hybridization techniques, between the *S. typhi* and *E. coli* K12 genomes (Falkow *et al.*, 1962).

It appears that Mu is a useful tool for the genetic analysis of *S. typhi*. Even though it can not plaque on wild-type strains, Mu can be used to induce mutations and promote intra- and interspecific genetic transfer. This opens many possibilities for the investigation of the virulence and pathogenicity of *S. typhi*. The isolation of strains on which Mu plaques also allows the analysis of other properties such as recombination, and DNA restriction and modification in this organism.

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