MALE SPECIFIC BACTERIOPHAGE IN *SHIGELLA FLEXNERI*

II. GROWTH CHARACTERISTICS OF PHAGE IN F⁺ *SHIGELLA**

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Multiplication of male specific bacteriophages, MS-2 and M12, has been demonstrated in F⁺ *S. flexneri*. These bacteriophages did not form any plaque on intact F⁺ *Shigella*, but the phage RNA could infect spheroplasts of *S. flexneri* with a similar plating efficiency as in its infection of *E. coli*.

It was also indicated that the efficiency of adsorption of MS-2 or M12 to intact F⁺ *Shigella* was very low; however, once the phage RNA was injected into the bacteria, phage replication occurred therein as well as in *E. coli* K12; the burst size was about 2,000 and the latent period was about 20 min., in both *E. coli* and *S. flexneri*.

No affirmative evidence for the multiplication and continuous release of RNA phages without killing the host bacterial cells, F⁺ *Shigella*, has been obtained.

INTRODUCTION

It is known that many *Shigella* strains were able to receive the sex factor from *Escherichia coli* male strains, and also transfer the F or fragment of *Shigella* chromosome to F⁻ bacteria (Luria and Burrous, 1957; Schneider and Falkow, 1964; Kitano, 1966).

Meanwhile, a number of bacteriophages containing RNA have been isolated by many investigators, since f2 phage was first discovered by Loeb and Zinder (1961). Generally, most of these RNA phages were able to establish infection specifically in *E. coli* male strain, but not in female one, excepting a single report by Feary, Fisher and Fisher (1963). Such male specificity of RNA phages may be closely related to a specific structure of pili formed on the surface of bacteria (Crawford and Gesteland, 1964; Brinton, Gemski and Carnahan, 1964).

Since MS-2 phage (Davis, Strauss and Sinsheimer, 1961) did not make any plaque on F⁺ *S. flexneri*, as described in a previous report (Kitano, 1966), it was needed to confirm whether or not the male specific phage was able to infect F⁺ *Shigella*. The present report describes details of MS-2 and M12 phages' multiplication in F⁺ *S. flexneri*, producing progeny particles therein as well as in the *E. coli* male strain.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains used were *E. coli* K12S, *E. coli* K12 W3747 F13, *S. flexneri* 2a E66, *S. flexneri* 2a E66-C3 F⁺ and *S. flexneri* 2a E66-C4 F⁺.

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Clones of F'^+ *S. flexneri* were obtained according to the method described in a previous report.

Bacteriophages: MS-2, M12 (Hofschneider, 1963), Q β and R164 phages were used in this experiment. M12 was kindly supplied from Dr. Hofschneider through Dr. Y. Hirota, and Q β from Dr. I. Watanabe. R164 was isolated from human feces. All phage lysates were prepared from infected *E. coli* K12S cultured in peptone glucose medium (PG) with the addition of one-twentieth volume of 5% yeast extract and one-hundredth of 1M $CaCl_2$.

Antiserum preparation: Antiserum against M12 phage was prepared as follows; about 2×10^{11} PFU per ml of the lysate was injected intravenously into a rabbit five times at three day intervals. After one week from the last injection, the animal was sacrificed and the serum separated. K value of this antiserum was about 200.

Preparation of bacterial spheroplasts: Bacterial spheroplasts were prepared according to Guthrie and Sinsheimer (1960). The bacteria were cultured in modified 3XD medium. At a log phase of the culture, showing an OD_{660} of about 0.5, 20 ml of bacterial culture was centrifuged for collecting the cells, then resuspended in 0.35 ml of 0.5 M sucrose plus 0.1 ml of 0.25 M Sigma Trizma Base buffer of pH 8.1. To the suspension were added 0.002 ml of 1% lysozyme (Sigma) and 0.002 ml of 4% EDTA then the mixture was kept standing for ten min. at room temperature. After becoming viscous, it was diluted with twenty times volume of nutrient broth used by Guthrie and Sinsheimer, using 3.8 g beef extract and 6.2 g polypeptone per liter instead of Difco Nutrient Broth. A half ml of 5 mg per ml protamine sulfate was added to the final spheroplast suspension.

RNA extraction from bacteriophages: RNA of the bacteriophages was prepared by the phenol method. An equal volume of cold phenol, saturated with water containing 1 mM EDTA and 1% bentonite (Kawade, personal communication), was added to the phage lysate, then the mixture was stirred for ten min. at room temperature. The aqueous layer was withdrawn after centrifugation, and the extraction with phenol was repeated three times. Two volumes of cold ethanol was added to the aqueous layer in the presence of 2% potassium acetate. The mixture was left standing for a few hr in the cold, and RNA was collected by centrifugation. The RNA was dissolved in 1 mM EDTA solution, followed by dialysis against 1 mM EDTA in the cold. The isolated RNA was stored at $-20^\circ C$ until use.

Phage titration: Titration of RNA phages were performed by using *E. coli* K12S as an indicator strain, unless otherwise noted. The soft agar composition for intact phage assay was 20 g polypeptone, 5 g NaCl, 2 g glucose, 7.5 g agar and 8.6 ml of 1 N NaOH per liter. For RNA-spheroplast system, nutrient broth plus 0.75% agar was used as soft agar. The basal agar plates were prepared with nutrient agar.

RESULTS

Sensitivity of F'^+ Shigella to RNA Phages

In a previous report it was shown that all clones of F'^+ *S. flexneri* seemed to be insensitive to MS-2 infection, as evidenced by the fact that the plating efficiency of MS-2 was less than 10^{-10} on F'^+ *Shigella* as compared with the high efficiency on the *E. coli* male strain. In the case of F'^+ *E. coli* converted from F^- strain by F-duction, the sensitivity against MS-2 was of the same order as that of the donor *E. coli*. The sensi-

tivity to other RNA phages, M12, Q β , and R164 was examined by using F⁺ *Shigella* strains. Clone E66-C3 or E66-C4 was inoculated on the plate after mixing with soft agar, and then one drop of phage lysate was spotted on the plate. As a control, *E. coli* K12 W3747 was tested at the same time. During incubation at 37 C, typical lytic regions were seen on W3747 culture; however no lysis was observed on the culture plate of E66-C3, which was a converted F⁺ strain of *S. flexneri*. On the other hand, some of faint lytic regions were detected on the plate of clone E66-C4. Hence it was concluded that most of F⁺ *Shigella* might be resistant to the RNA phages, but a minor population of F⁺ *Shigella* was sensitive to the RNA phages.

Infection of Phage RNA to Bacterial Spheroplasts

Infectious RNA has been isolated from many animal viruses and RNA phages, which has been able to infect animal cells or bacterial spheroplasts insensitive to infection with complete virus particles (Holland, McLaren and Syverton, 1959; Paranchych, 1963; Engelhardt and Zinder, 1964; Ben-Gurion and Ginzburg-Tietz, 1965; Kawade, personal communication). Therefore, the infection of *S. flexneri* with phage RNA was thought possible, when spheroplasts were used as an infecting system.

The phage RNA diluted in 1 mM EDTA was mixed with nine volumes of the spheroplast suspension, then the mixture was plated immediately and the plates were incubated at 37 C. The results obtained are shown in Table 1, which indicates obviously that the phage RNA can multiply in the spheroplasts of *S. flexneri* and produce progeny particles, even though the host cells have the F⁻ character. It is also observed that in *E. coli*

Table 1. Infectivity of viral RNA to spheroplasts of *E. coli* and *S. flexneri*

Source of RNA	Input phage unit per ml	Spheroplasts	Number of plaques per 0.1 ml*			
			Dilution of RNA			
			10 ⁻⁰	10 ⁻¹	10 ⁻²	10 ⁻⁰ +RNase
M12	6.8 × 10 ¹¹	K12S	59	20	2	0
		E66	220	19	0	0
MS-2	2.8 × 10 ¹²	K12S	104	49	10	0
		E66	c**	268	12	0

* Before infection, RNA were incubated for ten minutes with 1/10 volume of saline or RNase (100 µg/ml).

** confluent lysis.

spheroplasts the number of plaques was not proportional to the concentration of phage RNA. This may be due to the lower stability of spheroplasts of *E. coli* as compared to those of *S. flexneri*.

Adsorption Efficiency of RNA Phage to Bacteria

Since the phage RNA was able to infect the spheroplast preparations of F⁺ *S. flexneri* the failure of bacteria to support its growth might be explained by the lack of the adsorption site or receptor on the surface of the converted bacteria. Another possibility should be considered as such that the adsorbed phages did not go into the cells and no phage RNA replication occurred.

To answer this question, an adsorption experiment was performed in the following

way: A log phase culture of bacteria suspended in pre-warmed PG medium containing 0.002 M KCN was mixed with an equal volume of a M12 suspension pre-warmed at 37 C. The mixture was incubated at 37 C, and samples were taken out at various intervals. Free phages were separated by filtration through an HA type Millipore filter and assayed. As shown in Fig. 1, neither E66 nor E66-C3 clones adsorbed M12 phage. It seems likely that E66 could not adsorb M12 phage because of its F^- character. In the case of E66-C3, it was observed that bulk of F'^+ *S. flexneri* also failed to adsorb M12. However, the possibility cannot be excluded that a minor population of E66-C3 contribute to M12 infection and allows their multiplication in the cells.

Fig. 1. Adsorption efficiency of M12 to *E. coli* and *S. flexneri* strains.

The experimental procedure is given in the text. Each point indicates the titer of filtrable unadsorbed phage. ○—○ on W3747, moi=1.3; ●—● on E66 (F^-), moi=0.2; △—△ on E66-C3 (F'^+), moi=2.7; □—□ on E66-C3, moi=0.04.

Multiplication of RNA Phage in F'^+ S. flexneri

A log phase culture of K12S, W3747, E66, E66-C3 or E66-C4 cells was infected with M12 or MS-2, and then it was incubated for 90 min. at 37 C with aeration. The incubation was stopped by addition of chloroform and the titer was measured before and after treatment with lysozyme. The results are summarized in Tables 2 and 3, which demonstrate that the lysozyme treatment always gave higher titers than those of untreated cases except a single case of the E66 F^- strain. The increase of phage titer due to the

lysozyme treatment was 1.5 times in the case of W3747 and 700 times in the case of E66-C3, as shown in Tables 2 and 3. These results suggest that, in spite of phage multiplication in the cells, release of progeny particles might be difficult especially in the case of E66-C3. It was also considered that the small number of progeny phages in F'^+ *S. flexneri* might be due to a small burst size and a long latent period of RNA phages in the strains.

One step Growth of M12 in F'^+ Shigella

A log phase culture of W3747 or E66-C3 was collected and infected with M12 at a multiplicity of 0.1 in the former or 0.2 in the latter. They were resuspended in PG containing 0.002 M KCN and allowed to stand for ten min. at 37 C. At the end of

Table 2. Multiplication of M12 in *E. coli* and *S. flexneri*

Bacteria	Input titer of M12 (A)	Input ratio	Treatment*	Final titer of M12 (B)	Multiplication of M12 (B/A)	Effect of lysozyme $\left(\frac{C+L}{C}\right)$
K12S	1.7×10^7	0.07	C	1.3×10^{10}	760	17
	"	"	C + L	2.2×10^{11}	13000	
W3747	"	0.28	C	2.2×10^9	130	50
	"	"	C + L	1.1×10^{11}	6500	
E66	"	"	C	1.3×10^7	0.8	0.9
	"	"	C + L	1.2×10^7	0.7	
W3747	1.7×10^7	0.08	C	5.5×10^9	19.5	730
	"	"	C + L	4.0×10^{11}	1400	
E66-C3	"	0.06	C	6.0×10^8	2.1	14
	"	"	C + L	8.5×10^9	30	

* C: After the incubation for 90 minutes, a few drops of chloroform was added to the culture. C+L: After the chloroform treatment, added 50 μ g/ml lysozyme.

Table 3. Multiplication of MS-2 in *E. coli* and *S. flexneri*.

Bacteria	Input titer of MS-2 (A)	Input ratio	Treatment	Final titer of MS-2 (B)	Multiplication of MS-2 (B/A)	Effect of lysozyme $\left(\frac{C+L}{C}\right)$
W3747	1.0×10^8	0.25	C	2.8×10^{11}	2800	5
	$\times$	"	C + L	1.4×10^{12}	14000	
E66-C3	"	1.0	C	1.8×10^8	1.8	42
	"	"	C + L	7.5×10^9	75	
W3747	2.0×10^6	2.2×10^{-4}	C	2.3×10^9	1150	1.5
	"	"	C + L	3.4×10^9	1700	
E66-C3	"	6.7×10^{-3}	C	2×10^6	1	22
	"	"	C + L	4.4×10^7	22	
E66-C4	"	4.5×10^{-3}	C	2.2×10^7	11	40
	"	$\times$	C + L	8.7×10^8	440	

The experimental conditions are the same as in Table 2.

the adsorption period, one-tenth volume of anti-M12 serum, having a K value of 20, was added to the mixture and it was incubated further for five min. . To grow progeny phages, the mixture was incubated at 37 C after diluting 10^4 times in PG. At various time intervals, samples were taken and plated for titration before and after treatment with chloroform-lysozyme. As can be seen in Fig. 2, the average burst size and profile of one step growth curve were very similar between both the strains, W3747 and E66-C3.

Fig. 2-a

Fig. 2-b

Fig. 2. One step growth curve of M12 in *E. coli* K12, W3747 (a) and in *S. flexneri* 2a E66-C3 (b).

Input ratio is 0.1 in *E. coli* and 0.2 in *S. flexneri*, respectively. ○—○ before and ●—● after treatment with chloroform-lysozyme (50 μ g/ml).

Namely, the latent period was about 20 min. and the average burst size was about 1,000, and no appreciable difference was observed in the growth characteristics, except a slight delay of initiation of the second growth cycle in *S. flexneri*. During the latent period, a considerable fraction of the infective centers were insensitive to chloroform treatment, as shown in Fig. 2b, suggesting that the attachment of M12 to the bacterial surface might be a reversible one and such attachment caused a protection of M12 inactivation by the anti-M12 serum.

The fact that the burst size was similar between W3747 and E66-C3 seems to be incompatible with the results obtained in the multiplication experiment described above. However, considering that the amount of phages effectively participating in the infection of F^+ *S. flexneri* was small, as shown in Fig. 1, it was concluded that the multiplica-

tion of M12 in F^+ *S. flexneri* was actually of the same order as in *E. coli*.

Another question arises why the lysozyme treatment increased the number of progeny phages, as shown in the multiplication experiment. From the results of the one step growth curve, it seems probable that the second growth cycle had proceeded in 90 min. of incubation, and progeny particles of the second growth cycle accumulated in the cells. The lysozyme treatment may have helped the release of those intracellular particles produced in the second cycle.

Single Burst Experiment of M12 in F^+ Shigella

Since the growth curve and the average burst size of M12 were similar between *E. coli* and *S. flexneri*, single burst experiments were performed to know the accurate number of progeny phages produced by an infected bacterium.

A log phase culture of W3747 or E66-C3 was diluted with PG containing 0.002 M KCN to about 10^7 cells per ml, then infected with M12 at a multiplicity of 1,900 in the case of *E. coli* or 670 in the case of E66-C3, and followed by an adsorption at 37 C for ten min. Then free phages were removed by an incubation for another 5 min. in the presence of the anti-M12 serum. The sample was diluted in PG and divided into a number of fractions so as to contain less than one infected bacterium per tube. After

Table 4. Single burst experiment of M12 in *E. coli*

No. of tubes	No. of M12 per tube
25	<10
2	2.0×10^3
2	1.7×10^3
2	3.4×10^3
1	1.1×10^3
1	2.2×10^3
1	6.4×10^2
1	4.8×10^3
1	2.7×10^3
1	1.3×10^3
1	7.2×10^2
1	3.0×10^3
1	2.9×10^3

an incubation for 90 min. at 37 C, each tube was treated with chloroform-lysozyme, and one-tenth volume of each vial was plated. The results are summarized in Tables 4 and 5. As can be seen in Table 4, fifteen out of forty vials had less than ten plaque forming units. From this result, it was calculated according to Poisson distribution that the average number of infected bacteria per tube, n , was 0.47, the total number of progeny phages 3.3×10^4 , the total number of infected cells 18.8 and the average burst size 1.76×10^3 in *E. coli*. Also, from Table 5, the average yield was reckoned as follows: $n = 0.028$, the number of progeny phages, the total number of infected cells and the average burst size were 6.9×10^3 , 2.8 and 2.3×10^3 , respectively. It can be concluded that once F^+ *S. flexneri* E66 is infected with M12, the progeny phage production proceeds at the same rate as in *E. coli* W3747.

Table 5. Single burst experiment of M12 in *S. flexneri*

No. of tubes	No. of M12 per tube
97	<10
1	3.1×10^8
1	2.0×10^8
1	1.7×10^8

It is known that certain bacteriophages such as fd, fr or M13 can grow and produce progeny phages without killing the host bacteria (Hofschneider and Preuss, 1963; Hoffmann-Berling, Dürwald and Beulke, 1963; and Hoffmann-Berling and Máze, 1964). Since such a phenomenon was considered possible in the M12-F'⁺ *S. flexneri* system, another single burst experiment was carried out. Experimental conditions were similar as described above except that before the chloroform-lysozyme treatment a half of the sample of each tube was taken for further growing of bacteria. After the chloroform-lysozyme treatment with the other half, one-tenth volume of each sample was plated for phage titration. From Table 6 it is noticed that ten out of one-hundred tubes contained progeny phages with the average burst size of 1.76×10^3 . Four tubes contained both progeny phages and surviving bacteria. There has been no critical evidence that the bacterial growth depended on escape from killing by phage or on contamination of non-infected bacteria.

Table 6. Growth of F'⁺ *S. flexneri* in single burst experiment

No. of tubes	No. of M12 per tube	Growth of bacteria
44	<10	no
46	<10	yes
2	1.2×10^3	no
1	1.6×10^3	no
1	2.4×10^3	no
1	1.5×10^3	no
1	1.1×10^3	no
2	2.3×10^3	yes
1	1.6×10^3	yes
1	2.5×10^3	yes

DISCUSSION

In a previous report, F'⁺ *Shigella* did not show any plaque formation by RNA phage infection. Those results seemed as if the converted F'⁺ *Shigella* had no receptor site for RNA phage, in spite of the fact that the bacteria were specified as male cells by F-factor transfer from F'⁺ *E. coli*. The present report supports this concept using the phage RNA for infection of spheroplasts of F⁻ *S. flexneri*, and this assay system was as fully efficient as in the infection of those of *E. coli* male strain. Although the efficiency of adsorption is very small on F'⁺ *S. flexneri*, as shown in Fig. 1, once phage RNA is injected into the cells, the viral RNA can replicate in F'⁺ *S. flexneri* as well as in

E. coli. Therefore, the conclusion is that the RNA phage infection of F'^+ *S. flexneri* may be difficult because of incompleteness of the bacterial surface or receptor site. Only a minor population of bacteria participates in the RNA phage infection.

The same is true in the case of F'^+ *Proteus mirabilis* infected with MS-2 (Horiuchi and Aderberg, 1965). The burst size of MS-2 in *P. mirabilis* was similar to that in *E. coli*, but no plaque formation was observed in the former case. One difference was seen in time lag for the latent period in *P. mirabilis* which was a little longer than in *E. coli*.

At the present time there is no precise understanding of the low efficiency of infection of F'^+ *S. flexneri*; however, three possibilities are considered: 1) F'^+ *S. flexneri* may be able to form only labile or incomplete F-pili, 2) the fine structure of F-pili may be modified under the genetic control mechanism of *Shigella*, so that the receptor site is insufficient for accepting the RNA phage, 3) only a minor population of F'^+ *S. flexneri* may be able to form complete F-pili. Recent knowledges have supported that the F-factor specifies the formation of F-pili which represents a specific site for RNA phages adsorption onto male bacteria (Crawford and Gesteland, 1964; Brinton et al., 1964).

From the results of the single burst experiment, no critical evidence has been obtained that RNA phage can infect, without killing, the host cells and continues the phage production.

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