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Growth of Male-Specific Bacteriophage in *Pasteurella* Harboring F-Genotes Derived from *Escherichia coli*

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When either the F' *lac* or the F'*Cm* plasmid was transferred from *Escherichia coli* into *Pasteurella pseudotuberculosis*, the *P. pseudotuberculosis* (F') strains isolated formed plaques with both ribonucleic acid (RNA)-containing and deoxyribonucleic acid-containing male-specific phages. In contrast, strains of *P. pestis* harboring *E. coli* (F') plasmids did not form plaques with male-specific phages, although such strains permitted limited multiplication of phage MS2. The adsorption and burst size of MS2 were approximately the same in both species of *Pasteurella*, but the per cent of adsorbed MS2 that produced infective centers was much lower in *P. pestis* than it was in *P. pseudotuberculosis*. By use of a sib-selection technique of *P. pestis* (F') cells, we isolated a single clone that could form MS2 plaques. ^{32}P-labeled MS2 adsorbed equally to and its RNA penetrated equally into both the typical MS2-non-permissive *P. pestis* cells and the MS2-permissive *P. pestis* cells. No host modification occurred after growth of MS2 in *Pasteurella*. Our data suggest that typical strains of *P. pestis* inhibit the intracellular development of phage MS2.

In *Escherichia coli*, the presence of the sex factor, F, can result in the production by the cell of an F pilus, a tubular appendage considered to be essential for gene transfer via conjugation (2). The F pilus also functions in *E. coli* as a receptor site for male-specific phage (4). Since the F factor can be transferred to bacteria in several different genera (5, 10), the possibility of propagating male-specific phage of *E. coli* in different genera can be investigated. This possibility has been studied by Horiuchi and Adelberg (6), who found that MS2 phage could grow in, but not form plaques on, *Proteus mirabilis* (F') cells, and by Kitano (8), who reported that *Shigella flexneri* (F'*lac*) cells were insensitive to infection with MS2 phage. This report summarizes our observations on the growth of MS2 phage in strains of *Pasteurella pseudotuberculosis* (F') cells and *P. pestis* (F') cells.

MATERIALS AND METHODS

Media and cultural conditions. Cultures were grown in 5 ml of Brain Heart Infusion broth (BHI; Difco) in test tubes (18 by 150 mm) incubated in a slanted position on a reciprocal shaker (5-cm stroke, 100 excursions per min). Strains of *Pasteurella* were incubated overnight at 26 C, and 0.5-ml volumes were subcultured into 5 ml of BHI at 37 C. Usually, maximum F piliation (determined by MS2 sensitivity) occurred in 5 hr. With *P. pestis*, better results were obtained when the overnight culture was centrifuged and 2 ml of cells (suspended to their original volume in 0.001 M potassium phosphate, *p*H 7.4, containing 1% gelatin and 0.2% glucose) was subcultured into 5 ml of BHI for 5 hr at 37 C. Strains of *E. coli* were always grown at 37 C, and MS2-sensitive cultures were obtained by subculturing 0.2-ml volumes of the overnight cultures into 5 ml of BHI for 3 to 5 hr.

TY medium, composed of 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 10 g of NaCl, 222 mg of $CaCl_2$, and 2 mg of $MnCl_2 \cdot 4H_2O$ per liter and adjusted to *p*H 7.4, was used to propagate MS2 phage. Hard agar plates contained 25 ml of Blood Agar Base (Difco). Soft agar contained 0.7% agar and 0.002 M $CaCl_2$ in Heart Infusion broth (HIB; Difco).

Viable counts were made on Purple Broth Base (Difco) supplemented with 1.5% agar, 1.0% lactose, and 0.00125% triphenyl tetrazolium chloride. This medium permitted us to distinguish *P. pestis* colonies from *P. pseudotuberculosis* colonies as well as *lac*$^+$ colonies from *lac*$^-$ colonies.

The standard diluent for cells and for phage suspension was HIB-PO_4 (HIB mixed 1:1 with 0.033 M potassium phosphate buffer, *p*H 7.4).

Phage. MS2 phage was obtained from the stock collection in this laboratory. It was propagated in shaken flasks of TY-Ca^{2+} at 37 C with *E. coli* W1485 as host. The phage was usually added at a multiplicity of infection (MOI) of 0.1 after the culture had grown for 2 hr (about 10^9 cells/ml). Incubation was continued for 4 to 5 hr, at which time partial lysis of the culture occurred. Cells were removed by centrifugation, and the supernatant fluid was filtered through sintered glass. Phage was assayed by the agar-layer technique by using *E. coli* HfrC as the indicator. Lysates usually contained about 10^{11} plaque-forming units (PFU) per ml.

MS2 titer increase. A 0.5-ml volume containing approximately 10^9 cells per ml (grown for 5 hr as described) was added to 2 ml of TY-Ca^{2+} containing 10^5 MS2 PFU/ml. The mixture was incubated overnight at 37 C with gentle shaking and was diluted 1:100 in HIB-PO_4 containing 0.5% chloroform. After thorough mixing and settling, appropriate dilutions of the supernatant fluid were assayed for MS2. To establish a baseline titer of MS2, the same procedure was run with BHI in place of the cells.

MS2 adsorption. A 1-ml volume containing about 10^9 cells per ml was added to 4 ml of TY-Ca^{2+} containing about 10^7 MS2 PFU/ml. After 10 min of adsorption at 34 C, the mixture was filtered through sintered glass, and the filtrates were assayed for free phage. Control tubes in which BHI replaced the cells were always included to obtain the initial MS2 titer.

One-step growth curve. The time course of the growth of MS2 in *Pasteurella* was followed by one-step growth experiments. The adsorption procedure was carried out at 34 C as described; the MOI varied with the bacterial strain used. After 10 min of adsorption, free phage was removed by incubation with anti-MS2 serum for another 5 min. This mixture was diluted 10^{-2} in prewarmed BHI to obtain the first growth tube, which in turn was diluted 10^{-2} for the second growth tube. Both growth tubes were incubated statically at 34 C, and samples were plated on HfrC at 10-min intervals. With *E. coli* strains, the procedure was the same except that the growth tubes were more dilute and samples were plated at 2-min intervals.

Preparation of ^{32}P-labeled MS2 phage. The procedures described by Brinton and Beer (1) were followed in the preparation of radioactive MS2 phage except that the TP medium of Kawakami and Landman (7) was used for labeling. *E. coli* W1485 was grown for 16 hr on a shaker, diluted 1:40 into 5 ml of TP medium, and incubated at 35 C for 2 hr prior to the addition of 300 μCi of ^{32}P-phosphoric acid. After 1 hr, MS2 phage was added at a multiplicity of about 10 PFU/cell. Incubation was continued for 3 hr, at which time ribonuclease, deoxyribonuclease, and lysozyme were added at levels of 40 μg/ml. After 15 min of incubation, chloroform was added to aid in the release of phage. The lysate was clarified by centrifugation at 10,000 rev/min for 10 min. The clear supernatant fluid was diluted with 10 volumes of 0.15 M NaCl and was filtered through a membrane filter (HA; Millipore Corp.). The membrane was eluted with two 5-ml volumes of TY broth over a period of 16 hr at 5 C, and the eluate was dialyzed to remove free ^{32}P. The final preparation contained 6×10^{10} PFU/ml and 2×10^5 counts per min per ml.

Adsorption of ^{32}P-labeled MS2 phage. Phage was diluted to the desired concentration in TY broth containing 0.002 M $CaCl_2$ and was equilibrated at 35 C. At zero time, cells, grown as described for the production of F pili, were added so that the multiplicity was about 1.0. Adsorption mixtures were incubated statically in a water bath at 35 C. Samples were removed at desired times and were centrifuged in the cold at 12,000 $\times$ *g* for 10 min to sediment the cells. Samples of both the supernatant fluid and the cells after suspension to the original volume in cold diluent were counted for radioactivity in a Nuclear-Chicago scintillation counter. The scintillation fluid contained 1.0 g of 2,5-diphenyloxazole (Packard), 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Packard), and 0.166 g of Triton X-100 (Packard) per liter of deionized water.

Penetration of ^{32}P-labeled MS2 phage. To remove F pili and unpenetrated phage, adsorption mixtures were centrifuged, and the cells were resuspended in 3 ml of TY broth and blended at half speed for 2 min in a Servall Omni-mixer maintained in an ice-water bath. The blended mixtures were centrifuged immediately in the cold at 12,000 $\times$ *g* for 10 min. Samples of both the supernatant fluid and the resuspended cells were counted for radioactivity.

RESULTS

Sensitivity to male-specific phage. When the F'*lac* plasmid was transferred from *E coli* (F'*lac*) into *P. pseudotuberculosis*, the strain of *P. pseudotuberculosis* (F'*lac*) isolated was sensitive to the male-specific phage MS2 and could donate both the F'*lac* plasmid and its chromosome to other strains of *P. pseudotuberculosis* (9). In contrast, when we transferred the F'*lac* plasmid from *E. coli* (F'*lac*) or from *P. pseudotuberculosis* (F'*lac*) into *P. pestis*, the strains of *P. pestis* (F'*lac*) isolated could donate the F'*lac* plasmid to recipient strains of *E. coli* or *P. pestis* but could not donate chromosomal markers to other strains of *P. pestis* and were not sensitive to phage MS2. We extended this observation of MS2 resistance to include other male-specific phages and found the same results with the ribonucleic acid (RNA) phages Qβ, f2, and R17 and the deoxyribonucleic acid (DNA) phages f1 and M13. Several different strains of *P. pestis* containing F'*lac* or F'*Cm* would not form plaques with male-specific phages.

MS2 titer increase. If the *P. pestis* (F') strains contained some MS2-sensitive cells mixed with a majority of MS2-resistant cells, plaque formation would be undetected because of overgrowth by the MS2-resistant cells. Therefore, we used the more sensitive technique of measuring the increase in MS2 phage titer after overnight incubation of 10^5 PFU/ml with various strains of *P. pestis* (F'), *P. pseudotuberculosis* (F'), and *E. coli* (F') cells. Phage MS2 multiplied in strains of *P pestis* (F'), but permitted only a one- to three-log MS2 titer increase compared with the six- to seven-log MS2 titer increase observed with *P. pseudotuberculosis* (F') or *E. coli* (F') strains. We concluded that three different strains of *P. pestis* containing one of five different F' plasmids all permitted only a relatively small increase in titer of MS2 phage.

Parameters of MS2 development. To determine

TABLE 1. *Growth parameters of MS2 phage in Escherichia coli (F'), Pasteurella pseudotuberculosis (F'), and Pasteurella pestis (F') cells*

Strain	Plasmid	Per cent of MS2 adsorbed in 10 min	Infective centers as per cent of adsorbed MS2	MS2 latent period (min)	Estimated MS2 burst size[a] (PFU/cell)
E. coli					
23.10S	F'*lac*	96	70	34–36	2,600
K57	F'*Cm*	82	82	34–36	2,000
P. pseudotuberculosis					
YsD-20	F'*lac*	64	22.1	60–70	900
YsD-40	F'*Cm*	45	25.4	60–70	1,500
P. pestis (typical MS2-nonpermissive)					
YpA-28	F'*lac*	74	2.1	70–80	600
YpA-22	F'*Cm*	46	2.7	70–80	2,100
P. pestis (MS2-permissive isolates)					
YpA-46	F'*lac*	77	26.1	70–80	3,000
YpA-38	F'*Cm*	57	24.0	70–80	1,500

[a] Burst size was estimated from the results of one-step growth curves.

why the multiplication of MS2 phage was limited in F' cells of *P. pestis*, we compared various parameters of the growth of MS2 in *P. pestis* (F'), *P. pseudotuberculosis* (F'), and *E. coli* (F') cells (Table 1). Since the adsorption and burst size were approximately the same in all three species, the possibility was excluded that only a few *P. pestis* (F') cells could adsorb MS2 or that the reduced MS2 titer increase in *P. pestis* (F') strains resulted from a very small burst size. The latent period was 70 to 80 min in *P. pestis* (F') strains, 60 to 70 min in *P. pseudotuberculosis* (F') strains, and 34 to 36 min in *E. coli* (F') strains. Most significantly, the number of infective centers expressed as the per cent of MS2 adsorbed (at an MOI <1) was lower with typical strains (i.e., MS2-nonpermissive) of *P. pestis* (F') than with *P. pseudotuberculosis* (F') or with *E. coli* (F') strains. We concluded that MS2 could adsorb to typical *P. pestis* (F') cells but that most of the adsorbed phage particles either did not inject their RNA into the cell or, if they did, were inhibited from developing within most cells.

Isolation of MS2-permissive P. pestis. Our results suggested that, within the strains of *P. pestis* (F'), a small number of cells existed that did not inhibit the development of MS2. Starting with a strain (YpA-22) that showed a relatively high MS2 titer increase, we used a sib-selection technique (3) to isolate an MS2-permissive strain. Twenty tubes, each containing approximately 10 cells, were incubated overnight and tested for ability to permit an MS2 titer increase. Two of the subcultures showed a larger MS2 titer increase than did the parent population. From one of these two subcultures, we tested 20 single clones and found that seven formed MS2 plaques. One of these clones was labeled YpA-38 and was used for subsequent study.

As shown at the bottom of Table 1, the adsorption, latent period, and burst size were similar when MS2-permissive *P. pestis* was compared with typical MS2-nonpermissive *P. pestis*. However, the percentage of adsorbed phage that produced infective centers was about 10 times higher with the MS2-permissive cells than it was with the MS2-nonpermissive cells. Thus, YpA-38 differs from its MS2-nonpermissive parent, YpA-22, only in the observed increase in the percentage of adsorbed phage that produced infective centers.

Additional evidence that YpA-38 is an MS2-permissive cell was obtained by isolating an F^- strain from YpA-38 and showing that F'*lac* or F'*Cm* from strains of *E. coli* passed into it by conjugation resulted in F' recipients that were fully permissive to MS2 (e.g., YpA-46). On the other hand, transfer of F'*Cm* or F'*lac* from MS2-permissive strains to typical strains of *P. pestis* did not confer on them the ability to form MS2 plaques.

Penetration of MS2 RNA into P. pestis. To determine if our observed difference of infective centers was caused by the inability of the phage RNA to penetrate MS2-nonpermissive *P. pestis* cells, we measured the penetration of MS2 RNA labeled with ^{32}P into the MS2-permissive strain YpA-38 and into the MS2-nonpermissive strain YpA-17. The data in Table 2 show that the adsorption of phage and the penetration of labeled RNA into both strains was approximately

TABLE 2. *Penetration of MS2 RNA into P. pestis*

Strain	MS2 plaques	Not blended			Blended		
		Total counts/min		Amt of counts/minute adsorbed (%)	Total counts/minute		Amt of adsorbed counts/minute penetrated (%)
		Cells	Supernatant		Cells	Supernatant	
Expt. A							
YpA-38	+	29,200	22,800	56.0	18,600	5,700	63.7
YpA-17	—	28,850	20,550	58.4	16,700	5,700	57.9
Expt. B							
YpA-38	+	34,000	29,000	54.0	22,500	5,100	66.2
YpA-17	—	35,800	30,200	54.3	18,580	12,300	51.9

equal. The data in Table 2 represent two of our recent experiments. The data obtained from 12 separate experiments were analyzed by Student's *t* test, and the results supported our conclusion that there is no significant difference between YpA-38 and YpA-17 in adsorption or penetration of MS2 phage.

Although the phage could penetrate, no measurable reduction in viability or growth rate occurred in strain YpA-17 after incubation with phage MS2 (MOI = approximately 10). Under the same conditions, strain YpA-38 showed an 84% reduction in viability.

Absence of host modification of MS2 in Pasteurella. The availability of a strain of *P. pestis* fully sensitive to MS2 permitted us to compare the relative efficiencies of plating of MS2 grown in *E. coli* (F'), *P. pseudotuberculosis* (F'), or *P. pestis* (F'). Regardless of which species was used to propagate MS2, the efficiency of plating was 0.3 to 0.5 in *Pasteurella* compared with 1.0 in *E. coli*. We concluded that no modification of MS2 occurred after propagation in *P. pseudotuberculosis* (F') or *P. pestis* (F').

DISCUSSION

Failure of male-specific phages to form plaques on a male bacterial host has been noted before. *Proteus mirabilis* harboring F genotes was shown to have a low efficiency of MS2 adsorption (6). Mutants of *E. coli* have been described that are resistant to phage f2 and the related phage MS2 but remain sensitive to the unrelated RNA phage Qβ and to the DNA phage f1 (11). Adsorption of f2 to the F pilus occurred normally, but RNA injection was blocked. Walker and Pittard (12) described an *E. coli* mutant that was insensitive to MS2 phage at 42 C and suggested that the structure of the F pilus was altered at that temperature. Contrary to the usual implication of defective F pili, we found that MS2-nonpermissive *P. pestis* (F') strains adsorbed the phage and permitted penetration of the phage RNA equivalent to the phage RNA penetration in an MS2-permissive *P. pestis* (F') strain. We conclude that typical strains of *P. pestis* can prevent the intracellular development of MS2 but that these strains contain a small number of cells permissive to MS2 development. Based on the observation that nonpermissive strains of *P. pestis* (F') yielded approximately one-tenth the number of infective centers as did the permissive strains (Table 1), we expected to find that a typical nonpermissive population would contain 10% permissive cells. However, all of 100 single clones from each of two different *P. pestis* (F') populations were MS2-nonpermissive. Our selection of the MS2-permissive strain YpA-38 from strain YpA-22 suggested that at least 3.5% of the nonpermissive population was MS2-permissive. It is difficult to determine the percentage of MS2-permissive cells in a "typical" MS2-nonpermissive strain. In some of our strains of *P. pestis* (F'), it seemed to approach 10%, but in others it appeared to be less than 1%. We isolated such an MS2-permissive clone from *P. pestis* and demonstrated that it did form MS2 plaques. This MS2-sensitive isolate also formed plaques with the male-specific RNA phages Qβ, f2, and R17 and the male-specific DNA phages f1 and M13. Since all of these phages did not form plaques on typical *P. pestis* (F') strains, the mechanism that restricts the intracellular development of MS2 RNA may also affect the development of the DNA phages. The latter possibility has been supported by our recent demonstration that a male-specific DNA phage, f1, adsorbed equally well (approximately 80% in 40 min) to MS2-permissive *P. pestis* (F') cells and to typical MS2-nonpermissive *P. pestis* (F') cells. This possibility and the intracellular fate of MS2 RNA in *P. pestis* are currently under investigation.

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