ABORTIVE INFECTION BY BACTERIOPHAGE BF23 DUE TO THE COLICIN Ib FACTOR. I: GENETIC STUDIES OF NONRESTRICTED AND AMBER MUTANTS OF BACTERIOPHAGE BF23[1]

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BACTERIOPHAGE BF23 was isolated by FREDERICQ, and has been used to detect bacterial strains that are resistant to colicins of group E (FREDERICQ 1949). NISIOKA and OZEKI (1968) noted that BF23 is related to T5 in its properties—both phages display virtually identical gross morphologies, both require calcium ions for multiplication, both have a minimal latent period of about 35 minutes, and both are restricted in their growth in cells of *Escherichia coli* carrying colicinogenic factor Ib (*Col*Ib$^+$ cells). Furthermore, these two phages undergo phenotypic mixing and, as we will report in a later publication, they readily recombine genetically with one another.

Restriction of the growth of BF23 by colicinogenic factor Ib was first observed by STROBEL and NOMURA (1966). Their work was subsequently extended by NISIOKA and OZEKI (1968). Our present knowledge of this restriction may be summarized as follows. (1) The efficiency of plating of BF23 on *Col*Ib$^+$ cells (restrictive host) is less than 10^{-6} when compared to that on *Col*Ib$^-$ cells (permissive host). (2) The adsorption of the phage and the injection of its DNA are both normal even when restrictive cells are used as the host. (3) In restrictive host cells the injected DNA is neither broken down into acid-soluble materials nor fragmented into small molecular species as judged by centrifugation in a sucrose density gradient. However, phage DNA is not synthesized. (4) Infection by the phage causes the degradation of host DNA in both the restrictive and permissive hosts. (5) *Col*Ib$^+$ cells lyse 15 minutes after infection by wild-type BF23 whereas *Col*Ib$^-$ cells lyse at the usual time of about 45 minutes after infection. This unusual lysis of *Col*Ib$^+$ cells is called "early abortive lysis" and requires the synthesis of phage-specific proteins other than lysozyme. (6) The rare plaques that do form on *Col*Ib$^+$ cells infected with a preparation of wild-type BF23 are not due to the phenomenon of "host-controlled modification" as observed with phage λ in certain *E. coli* strains (ARBER and LINN 1969) and with nonglucosylated T-even phages in cells lysogenic for P1 (REVEL 1967; REVEL and GEORGOPOULOS 1969). Rather, they result from a spontaneous mutation in the BF23 genome itself. These mutant phage, which can grow on *Col*Ib$^+$ cells, are recessive to wild-type phage in determining early abortive lysis.

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It has been proposed that bacteriophage infections cause structural alterations in the cell envelope of the host (PUCK and LEE 1954; 1955; SILVER 1965; FRANKEL *et al.* 1968) and that incomplete alteration of the structure of this cell envelope may result directly or indirectly in abortive infection. This proposed mechanism may be the cause of the abortive infections that occur when T4*rII* mutants infect λ-lysogens (SEKIGUCHI 1966) and when T4 or T2 infects ω-lysogens (PIZER *et al.* 1968). The infection of *Col*Ib$^+$ cells with wild-type BF23 may also cause a structural alteration of the cell envelope of the host which then leads to early abortive lysis.

With the above considerations in mind, we have focused our attention on the relationship between growth of BF23 and structural alterations of the host cell envelope. For this work, we have isolated mutants that can overcome the restriction by colicinogenic factor Ib and can therefore grow in *Col*Ib$^+$ cells. We have characterized these mutants genetically and biochemically. This paper presents the genetic features of one of our nonrestricted mutants in relation to those of a series of amber mutants of BF23 which we have also isolated. Evidence is presented which shows that the mutation that gives rise to the nonrestricted mutant as well as one of the amber mutations are both located within the 8% segment of BF23 DNA that is transferred to the host cell first (see RESULTS). We designate this segment the first-step-transfer or FST segment by analogy with T5 (MC CORQUODALE and LANNI 1964; LANNI, MCCORQUODALE and WILSON 1964; LANNI 1968).

MATERIALS AND METHODS

Media: Nutrient broth (NB) was prepared according to ADAMS (1959) and adjusted to pH 7.2 with NaOH. Nutrient broth containing 10^{-3} M calcium chloride is designated NB-Ca. Plating agar and soft agar contained 1.1 and 0.7% dehydrated agar (Difco Bacto-Agar), respectively, in nutrient broth. TGAB medium for bacterial growth contained 0.1 M Tris-HCl buffer at pH 7.4, 0.02 M NaCl, 0.003 M Na_2SO_4, 2×10^{-6} M $FeSO_4$, 0.001 M $MgCl_2$, 6.4×10^{-4} M potassium phosphate, 10^{-4} M $CaCl_2$, 0.02 M NH_4Cl, 0.5% glucose, and 30 μg per ml of each of 18 amino acids (Arg, Lys, His, Try, Tyr, Phe, Pro, Gly, Ala, Val, Leu, Ileu, Ser, Thr, Cys, Met, Asp, Glu). TGAP medium for the growth of phage had the same composition as TGAB except that 10^{-4} M $CaCl_2$ and 10^{-3} M $MgCl_2$ were replaced by 10^{-2} M $MgCl_2$ and gelatin was added to give a final concentration of 100 μg per ml. The buffer used for the resuspension of bacteria and for the adsorption of phage had the same composition as TGAP except that glucose, NH_4Cl, and the 18 amino acids were omitted.

Bacterial strains: The bacterial strains used in the present experiments are given in Table 1. *Salmonella typhimurium* LT2*cys*36*Col*Ib$^+$(P9) was kindly supplied by Dr. R. C. CLOWES. For the isolation of *E. coli* strains resistant to colicin Ib, *S. typhimurium* LT2*cys*36*Col*Ib$^+$(P9) was stabbed onto a nutrient agar plate and incubated overnight at 37°C. The colony that had formed was killed with chloroform vapors and then was overlaid with soft agar containing a colicin-sensitive strain. Resistant cells, which form colonies within the area into which colicin Ib had diffused from the original *S. typhimurium Col*Ib$^+$ colony, were picked and purified at least three times by single-colony isolation. In all experiments, colicin-resistant cells were used to avoid complications that may be introduced by colicin molecules themselves. Colicin-sensitive and colicin-resistant cells showed no difference in adsorption and growth of BF23.

For the development of the desired colicinogenic strains, transfer of colicinogenic factor Ib to appropriate recipient strains of *E. coli* was carried out by the procedure of OZEKI and HOWARTH (1961) using *S. typhimurium* LT2*cys*36*Col*Ib$^+$(P9) as the donor strain.

Bacteriophages: Samples of wild-type BF23 were obtained from both Drs. T. NISIOKA and M. NOMURA and were indistinguishable from one another in terms of their restriction of growth in *Col*Ib+ cells. A single plaque was isolated and this clonal phage was used as our wild-type strain of BF23 for all the experiments described in this paper.

For the isolation of nonrestricted mutants (designated *h*, for their *h*ost range characteristics), about 10^7 wild-type phage, which originated from an individual plaque, were plated on *E. coli* W3110*Col*Ib+ cells. Rare plaques that formed were picked and purified at least three times on *E. coli* W3110*Col*Ib− by single-plaque isolation. About 100 independent mutants were obtained in this manner. One of these mutants, designated *h*1, was used as a representative mutant in the work presented in this communication.

Amber mutants of BF23 were obtained by treatment of phage-infected permissive (CR63*su*+*Col*Ib−) or nonpermissive (W3110*su*−*Col*Ib−) cells with 5-bromo-deoxyuridine (BUDR) essentially according to FERMI and STENT (1962). Host cells were grown to about 7×10^7 per ml in TGAB, harvested by centrifugation, washed once in phage adsorption buffer, and resuspended in the same buffer at about 1.3×10^8 per ml. After starving the cells for 45 min by incubation without aeration at 37°C, wild-type phage at an input ratio of six per cell and BUDR were added. The phage were allowed to adsorb for 15 min, after which the buffer was converted to TGAP by the addition of glucose, ammonium chloride, and amino acids. The infected complexes, now at about 1×10^8 per ml of TGAP containing 175 μg of BUDR per ml, were incubated for 30 min at 37°C with gentle aeration. The complexes were then diluted 2500-fold into TGAP containing 200 μg of thymidine per ml and quickly divided into many separate samples. After a further incubation period of 120 min at 30°C, a drop of chloroform was added to each sample to ensure that all the cells were lysed. Amber mutants among the progeny were selected by adsorbing progeny phage onto CR63*su*+*Col*Ib− and plating on a mixed indicator consisting of 1 part of CR63*su*+*Col*Ib− and ten parts of W3110*su*−*Col*Ib−. Small, turbid, and other abnormally appearing plaques were picked and tested for their ability to grow on CR63*su*+*Col*Ib− and on W3110*su*−*Col*Ib−. The mutants capable of growing on CR63*su*+*Col*Ib− but not on W3110*su*−*Col*Ib− were further purified by single-plaque isolation.

Preparation of phage stocks: Stocks of necessary strains of phage were prepared by plating for confluent lysis on suitable indicator strains. About 10^4 phage were preadsorbed for 15 min at room temperature to indicator cells growing exponentially in NB·Ca, and plated with two drops of an overnight culture of the same indicator strain. After overnight incubation at 37°C, each plate was flooded with 6 ml of NB·Ca in order to suspend the phage that had formed. The phage suspension was collected by aspiration with a Pasteur pipette, treated with chloroform, and then centrifuged at 5000 rpm for 15 min to remove bacterial debris. The titer of the resulting phage suspension was usually about 1 to 2×10^{11} per ml. Wild-type phage and *h* mutants were prepared on W3110*su*−*Col*Ib−, whereas amber mutants were prepared on CR63*su*+*Col*Ib−.

Further purification of the phage particles was carried out by differential centrifugation. The particles were sedimented by centrifugation for 120 min at 15000 rpm, resuspended in phage adsorption buffer, and centrifuged again for 15 min at 5000 rpm. This procedure was repeated once, and the supernatant from the final centrifugation was considered as the purified phage suspension.

Preadsorption procedure for assay of phage: When BF23 was plated directly with an indicator strain, the sizes of the plaques that later formed were heterogeneous. Plaques of uniform size develop when the phage are preadsorbed to host cells prior to plating. To do this, the phage to be assayed were mixed at an appropriate dilution with indicator cells growing exponentially (2×10^8 per ml) in NB·Ca. After 15 min at room temperature to allow adsorption of the phage, the infected cells were plated with two drops on an overnight culture of the same indicator strain. Other techniques employed in these experiments, but not described, are detailed by ADAMS (1959).

Complementation tests among amber mutants: The amber mutants were preliminarily assigned to individual cistrons on the basis of spot-complementation tests. The procedure employed for these tests was essentially the same as that described by FATTIG and LANNI (1965). One drop (approximately 0.05 ml) of a suspension of one of the mutants (at about 5×10^7 per ml of

NB·Ca) was applied to pre-dried plates seeded with W3110*su*⁻*Col*Ib⁻ cells and allowed to soak in thoroughly. One drop of the other phage to be tested was then applied in such a manner that the two areas on the plate where the spots were applied mutually overlapped about one-third of their areas. Lysis of the nonpermissive host cells in the overlap area, after overnight incubation at 37°C, was taken as evidence that complementation did occur between the two mutants tested. As a control, each mutant was spotted against itself.

Recombination tests of amber mutants: One volume of an overnight culture of CR63*su*⁺*Col*Ib⁻ in NB was inoculated into 100 volumes of fresh NB, and the bacteria grown to a concentration of 2.0×10^8 per ml. The cells were harvested by centrifugation for 10 min at 5000 rpm at 2°C, and resuspended at a concentration of 2×10^9 per ml in ice-cold NB containing 2×10^{-3} M KCN and 10^{-2} M $MgCl_2$. The resuspended cells were incubated for 5 min at 37°C, after which an equal volume of a mixture of parental phages, each at 1.0×10^{10} per ml of NB containing 10^{-2} M $MgCl_2$, was added. Adsorption of the phage was allowed to proceed for 15 min at 37°C without aeration and the infected complexes then quickly diluted 1000-fold into ice-cold NB·Ca. One volume of this diluted culture was added to 100 volumes of NB·Ca prewarmed to 37°C and incubated for 140 min with aeration. After this last incubation period, several drops of chloroform were added to assure complete lysis of the cells. The lysates, after suitable dilution, were assayed on CR63*su*⁺*Col*Ib⁻ for the total number of progeny, and on W3110*su*⁻*Col*Ib⁻ for the number of wild-type recombinants produced. For each recombination test, the number of infected cells and unadsorbed phage present at the beginning of the 140 min incubation period was determined on CR63*su*⁺*Col*Ib.⁻ Under the conditions described, the efficiency of phage adsorption was more than 98%. As controls, each parental phage was used to infect CR63*su*⁺*Col*Ib⁻ cells at an input ratio of ten which was equal to the total input ratio used in each recombination test. Total progeny phage and wild-type phage formed in these controls were assayed on CR63*su*⁺*Col*Ib⁻, and

TABLE 1

Bacterial strains

Strain	Genetic markers	Growth of BF23 strains (+ growth supported) (- growth not supported)			
		h^+am^+	h^-am^+	h^+am^-	h^-am^-
Derivatives of *Escherichia coli* K12:					
W3110 (wild type)	su^-·ColIb^s·ColIb$^-$	+	+	-	-
W3110	su^-·ColIb^r·ColIb$^-$	+	+	-	-
W3110	su^-·ColIb^r·ColIb$^+$	-	+	-	-
CR63	su^+·ColIb^s·ColIb$^-$	+	+	+	+
CR63	su^+·ColIb^r·ColIb$^-$	+	+	+	+
CR63	su^+·ColIb^r·ColIb$^+$	-	+	-	+
Derivative of *Salmonella typhimurium* LT2	cys36·ColIb$^+$(P9)				

on W3110*su*⁻*Col*Ib⁻, respectively. Since the fraction of wild-type phage that formed to total progeny phage was always less than one percent in these controls, the recombination values calculated from the crosses would not be significantly altered if this small correction were applied.

Crosses between the nonrestricted mutant and amber mutants: The procedure employed for crosses between the nonrestricted mutant, *h*1, and various amber mutants was identical to that used for crosses between amber mutants except that different indicator strains were used to detect the various recombinants formed. As shown in Table 1, all types of expected recombinants can be quantitatively determined with appropriate combinations of the various indicator strains. Two methods were used for determining recombination frequencies. The first of these was "differential plating," which is based on the inability of wild-type recombinants to grow on W3110*su*⁻*Col*Ib+ and their ability to grow on W3110*su*⁻*Col*Ib⁻. Since one of the parental strains, *h*⁻*am*+, can grow on both W3110*su*⁻*Col*Ib⁻ and W3110*su*⁻*Col*Ib+, the difference between the number of plaques formed on W3110*su*⁻*Col*Ib⁻ and on W3110*su*⁻*Col*Ib+ corresponds to the number of wild-type recombinants formed. Similarly, the difference between the number of plaques formed on CR63*su*+*Col*Ib+ and on W3110*su*⁻*Col*Ib+ will give the number of double marker recombinants, *h*⁻*am*⁻. The second method was "single-plaque characterization" in which all progeny phage were plated first on CR63*su*+*Col*Ib⁻. Individual plaques were then picked with a toothpick and stabbed onto four plates each seeded with one of the four indicator strains, CR63*su*+*Col*Ib⁻, CR63*su*+*Col*Ib+, W3110*su*⁻*Col*Ib⁻, and W3110*su*⁻*Col*Ib+. The genotype of each plaque was identified according to the growth patterns shown in Table 1 and the number of plaques having a given genotype determined. In practice, the former method is much easier than the latter but the results obtained from the two procedures are essentially the same.

Recombination tests between first-step-transfer segments and genomes of superinfecting phage: One volume of an overnight culture of CR63*su*+*Col*Ib⁻ in TGAB was added to 100 volumes of fresh TGAB and grown to 2.5×10^8 per ml. One volume of this logarithmic cell culture was harvested by centrifugation for 10 min at 5000 rpm at 2°C, and the sedimented cells washed once with a half volume of ice-cold adsorption buffer. The washed cells were then resuspended in the same buffer at a concentration of 4.0×10^9 per ml and incubated for 15 min at 37°C. The parental phage chosen to supply the first-step-transfer segment was then added to the preincubated host cells at a ratio of 6 phage per cell. An adsorption period of 15 min at 37°C was permitted during which time the adsorbed phage transferred their FST-DNA to the host cells. After this adsorption period, the culture was quickly chilled in an ice bath, and the phage–bacterium complexes were mechanically sheared. For chilling and subsequent shearing, one volume of the infected culture was added to one volume of ice-cold adsorption buffer in a 50 ml blendor cup (Sorvall Omnimixer) immersed in an ice bath (0°C). The phage–bacterium complexes were then sheared for a total of 12 min (three 4-min periods) at the maximum speed of the Omnimixer. The sheared complexes, containing the FST-DNA but not the rest of the phage DNA, were sedimented for 10 min at 5000 rpm in order to remove free phage particles, and were resuspended in adsorption buffer to a concentration of about 4×10^9 per ml. Superinfection of these FST-infected cells was carried out by first equilibrating them for 5 min at 37°C and then adding the superinfecting phage at an input ratio of 6.5 to 7.5 per FST-complex. After an adsorption period of 15 min, the superinfected cells were diluted 100-fold with adsorption buffer and one volume of this diluted suspension was then inoculated into 100 volumes of TGAP medium. The resulting suspension was incubated for 140 min at 37°C, after which a few drops of chloroform were added to ensure lysis of the cells. As a control, buffer was added instead of superinfecting phage. Recombinants that formed were assayed by the procedure described above. The number of plaque-formers and unadsorbed phage was determined after the primary infection and after the superinfection by taking appropriate samples at each operational step.

Under the conditions employed, mechanical shearing by the Omnimixer did not result in any significant killing of uninfected cells.

RESULTS

Complementation and recombination tests among BF23 amber mutants: As the

TABLE 2

Grouping of amber mutants and recombination between selected mutants

Map segment	Representative mutant in each gene	Number of mutants in each gene	Per cent recombination* of the representative mutants with:						
			am57	am111	am63	am91	am141	am218	h1
I	am57	1	-						21.0
II	am111	2	53.6	-					45.4
II	am63	2	54.9	8.7	-				43.0
III	am91	7	52.1	44.5	50.4	-			33.0
III	am141	6	48.8	48.0	44.0	13.0	-		38.7
IV	am218	1	46.4	38.8	40.3	48.9	42.5	-	45.0
IV	am43	2	50.4	44.0	33.7	56.1	49.0	12.1	46.2
IV	am69	1	53.6	36.6	40.0	52.9	37.8	17.1	42.6
IV	am103	1	48.9	41.3	42.1			20.5	47.0
IV	am7	4	53.7	39.4	41.3	56.4	59.1	24.4	36.6
IV	am165	2		45.1	35.6			30.6	32.2
IV	am151	2	49.3			54.8			35.6
IV	am178	1	47.5			45.2			37.0
IV	am1	4	48.7			55.0	50.0		33.0
IV	am123	1	52.4			46.0		39.1	31.2
IV	am182	2							34.3
IV	am56	2						47.6	25.8
IV	am107	8	55.1	48.5	48.4	46.9	51.4	43.3	39.9
IV	am119	8	58.7			51.8		44.3	41.9
IV	am115	1	54.3			48.3			30.0
IV	am105	2	45.6					44.9	36.8
IV	am133	1	48.7			48.7			37.8
IV	am132	6	49.8	50.4	58.1	47.3		49.9	32.5
IV	am177	2	46.1	46.8	47.6	43.9		41.3	26.4
IV	am70	3	44.9	47.4	50.4	45.8	56.8	41.4	28.5
IV	am159	2	40.4	54.1	64.4	56.3			31.4
IV	am204	2	46.9	66.9	65.4	48.5			28.4
IV	am158	1	48.0	58.0	62.4	51.3	54.0	48.9	23.5

* Percent recombination is defined as

$$\frac{2 \times \text{titer on W3110}su^{-}Col\text{Ib}^{-}}{\text{titer on CR63}su^{+}Col\text{Ib}^{-}} \times 100.$$

first step in constructing a genetic map of BF23 spot-complementation tests between pairs of amber mutants were carried out in order to group those mutations affecting the same function into the same gene and those mutations affecting different functions into different genes. Seventy-seven mutants out of the approximately 100 mutants that had been isolated were assigned to 28 complementation groups. The remaining mutants had various undesirable properties, which

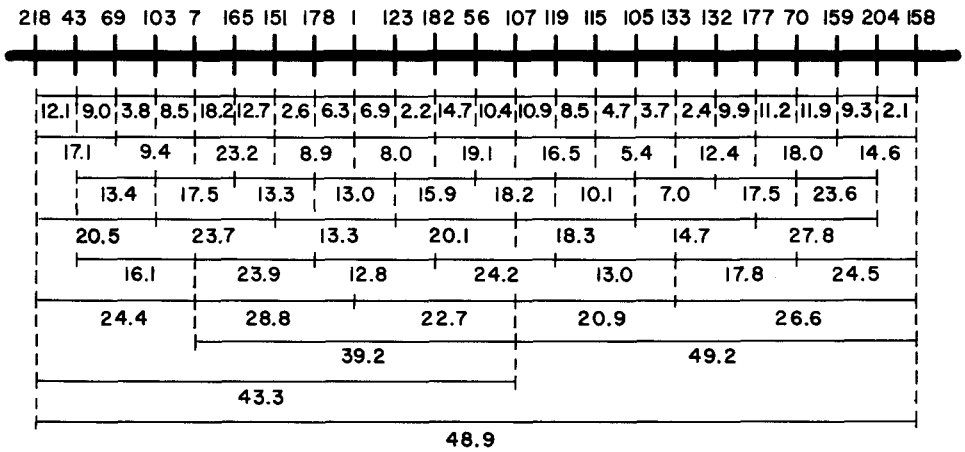

FIGURE 1.—Map of 23 genes in segment IV.

are not reported here, and we did not use them in subsequent experiments. Table 2 lists the 28 complementation groups, the number of mutants assigned to each group or gene (column 3), and the representative mutant for each gene (column 2). As will be seen later, the assignments of the isolated mutants are distributed widely over the BF23 chromosome. However, the fact that more than 70% of the genes identified contain only one or two mutations indicates that these 28 complementation groups may not be sufficient for the construction of the complete genetic map of BF23.

The results of two-factor crosses between representative amber mutants of each gene are summarized in Figure 1 and columns 4 through 9 of Table 2. Several crosses yielded maximal recombination frequencies (more than 40%), for example, all crosses between *am*57 and any other mutant, all crosses between either *am*91 or *am*141 and any other mutant, and all crosses between either *am*63 or *am*111 and any other mutant. The recombination frequencies given in Table 2, therefore, divide the genetic map into four possible segments or linkage groups, namely, segment I (*am*57), segment II (*am*111 and *am*63), segment III (*am*91 and *am*141), and segment IV (*am*218 to *am*158), as shown in column 1.

Figure 1 shows the relative position of 23 genes in segment IV. When attempts were made to order these genes from the data of two-factor crosses, it was difficult to decide the relative position of some genes because of the high recombination frequencies or the nonadditive map distances they gave with the closest mutant to them that we had. Such examples may be seen in crosses between *am*165 and *am*7 or *am*103, and between *am*178 and *am*123 or *am*182. Hence, three-factor crosses were performed in order to determine the relative positions of these genes. Three double mutants, *am*7–*am*1, *am*1–*am*107, and *am*107–*am*70, were isolated from crosses between the appropriate single mutants. The results of crosses between these double mutants and the single mutants corresponding to segment IV are shown in Table 3. When the double mutant, *am*7–*am*1 was used, *am*165, *am* 151, and *am*178 were mapped between *am*7 and *am*1; and *am*218, *am*43,

TABLE 3

*Three-factor crosses of mutants in segment IV**

Single mutant	Double mutant		
	am1-am7	am1-am107	am107-am70
am218	19.7	27.6	31.3
am43	13.7		
am69	6.2		
am103	6.0		
am7	< 0.1	23.4	26.9
am165	4.2	15.6	19.2
am151	2.3	8.7	
am178	2.1	7.2	
am1	< 0.1	0.0	14.7
am123	5.3	2.9	17.6
am182	7.0	1.5	12.7
am56	14.5	2.9	6.7
am107	22.0	0.0	0.0
am119		9.0	2.8
am115		13.9	4.2
am105		13.4	4.6
am133		14.4	3.0
am132		16.0	3.3
am177		20.0	2.9
am70	35.8	21.8	0.0
am159			8.4
am204			15.4
am158	43.5		15.8

* Numerical values represent the frequency (in percent) of formation of wild type and triple markers as defined in footnote (*) to Table 2.

*am*69, and *am*103 were located outside of the *am*7 through *am*1 region. Similarly, *am*56, *am*182, and *am*123 were mapped between *am*1 and *am*107 utilizing the double mutant *am*1–*am*107. These results and the data from two-factor crosses also show that *am*218 and *am*158 are at opposite ends of segment IV.

Two things may be pointed out from the assigned map positions of the 23 genes in segment IV (Figure 1). First, recombination frequencies show good additivity up to 18 to 20%. This additivity permits an estimate of 180 recombinational units for the genetic length of section IV. Second, maximal recombination values can be obtained in crosses between two mutants even in segment IV if they are sufficiently far apart. For example, *am*218 crossed with *am*107 gives 43.3% recombination, and indicates that any two genes must be separated by about 100 recombinational units in order to yield such maximal values.

TABLE 4

Characteristics of nonrestricted mutant h*1*

Strain	Efficiency of plating on		Transmission efficiency	Lysis time after infection (min)	
	W3110*Col*Ib⁻	W3110*Col*Ib⁺		W3110*Col*Ib⁻	W3110*Col*Ib⁺
wild	(1.0)	10^{-6}	0.05 ~ 0.08	50	15
*h*1	(1.0)	~0.7	1.0	50	50

Characterization of the nonrestricted mutant, h*1:* STROBEL and NOMURA (1966) and NISIOKA and OZEKI (1968) reported the isolation of nonrestricted mutants of BF23 that can grow on *Col*Ib⁺ cells. We have isolated about 100 mutants of this type and have selected one of them, *h*1, as a representative used in the work presented in this communication. Some of the properties of this mutant are presented in Table 4. If *h*1 is grown first in *Col*Ib⁻ cells and then in *Col*Ib⁺ cells, its growth is not restricted in the latter host. This growth behavior indicates that *h*1 is not the result of host-controlled modification, but rather, that *h*1 arises from a mutation in the phage genome. This result confirmed previous observations in other laboratories. The efficiency of plating of *h*1 on *Col*Ib⁺ cells was about 0.7 compared to that on *Col*Ib⁻ cells. We note that the efficiency of plating of our other *h* mutants varied from 1.0 to 0.01 and was characteristic for each mutant (MIZOBUCHI, unpublished results).

When *h*1 infects *Col*Ib⁺ cells and plaque-forming units are assayed on *Col*Ib⁻ cells, every infected *Col*Ib⁺ cell produces at least one progeny phage, which means that the transmission efficiency of *h*1 is 1.0. On the other hand, the transmission efficiency of wild type is 0.05 to 0.08. This means that 5–8% of a population of *Col*Ib⁺ cells were able to produce at least one progeny phage when infected with wild-type phage. Mixed infection of *Col*Ib⁺ cells with wild-type and *h*1 showed a transmission efficiency of about 0.1 to 0.15. This result indicates that the *h* mutation is recessive to wild type, in confirmation of the observations of NISIOKA and OZEKI (1968).

Recombination between h*1 and amber mutants:* Two-factor crosses between *h*1 and various amber mutants were carried out in CR63*su*⁺*Col*Ib⁻ and the progeny phage from these crosses were assayed either by the differential plating method or by the single-plaque characterization method. The results of these crosses, as determined by the differential plating method, are presented in the last column of Table 2. *am*57 gave the lowest recombination frequency (21%) of all mutants tested. Crosses between *h*1 and any mutant in the *am*158 through *am*177 region of segment IV (see Figure 1) yielded 24–30% recombination, but the lack of additivity in these values suggested to us that two-factor crosses between *h*1 and amber mutants failed to determine precisely the relative position of the *h*1 mutation in the BF23 chromosome.

Location of the h*1 mutation on the chromosome; recombination between FST-segments and superinfecting phage genomes:* The previous report by NISIOKA and OZEKI (1968) that BF23 and T5 have similar physiological properties, sug-

gested to us that BF23 injects its DNA in a two-step manner, first-step-transfer (FST) followed by the injection of the rest of the DNA, as does T5 (LANNI 1968; MCCORQUODALE and LANNI 1964; LANNI, MCCORQUODALE and WILSON 1964). We have shown that this is indeed the case and that the FST-DNA of BF23 is about 8% of the total phage DNA as judged by sedimentation of the ^{32}P-FST-DNA (MCCORQUODALE and STALLCUP, unpublished) and by the fraction of shear-resistant ^{32}P transferred to host cells from ^{32}P-labeled BF23 under conditions that do not permit protein synthesis. LANNI, LANNI and TEVETHIA (1966) and LANNI (1969) reported the genetic analysis of conditional-lethal mutants that were defective in genes located within the FST segment of T5. Genes could be assigned to the FST-segment on the basis of whether or not a given mutant could be complemented by the wild-type FST-DNA of T5 in a nonpermissive cell or could recombine with the FST-DNA to yield some wild-type progeny. Thus, it was of interest to determine by this method which of our mutants were located in the FST segment of BF23.

CR63*su*$^+$*Col*Ib$^-$ cells were infected with wild-type BF23 under conditions that permitted only the FST segment to be transferred to the host cells. The complexes were then sheared mechanically and superinfected with the mutant to be examined. The progeny phage that resulted after a suitable growth period were assayed on appropriate indicator strains. If the FST segment could recombine with the superinfecting phage genomone, and if the defective gene of the superinfecting phage were located in its FST segment, a significantly larger proportion of wild-type progeny would be expected from such superinfections when compared to a nonsuperinfected control. Conversely, if the defective gene of the superinfecting phage were not located in its FST segment, or if the FST segment could not recombine with the superinfecting phage genome, the proportion of wild-type progeny phage produced in the superinfected cells would be, at most, equal to that in the nonsuperinfected control.

In order to establish optimal experimental conditions for recombination experiments involving FST fragments, we examined the effect of the multiplicity of superinfection by the two amber mutants, *am*57 and *am*91, on such recombination. The results are presented in Table 5 and Figure 2. Mechanical shearing of phage–bacterium complexes formed at an input ratio of 6 phage per cell under conditions that allowed only the FST-DNA to be transferred from wild-type BF23 to CR63*su*$^+$*Col*Ib$^-$ decreased the plaque-forming units to less than 1% of the unsheared control. The fraction of the initial cells that could form colonies after shearing was about 5%. Thus, the injection of just the FST-DNA of BF23 kills the host cells but does not produce any progeny phage. Fifty to 70 percent of the FST-infected cells could be successfully superinfected as measured by plaque-forming ability. The decrease in plaque-forming units after superinfection may be at least partly due to inactivation of phage–bacterium complexes during the centrifugation and resuspension steps used to remove free phage.

At multiplicities of superinfection up to about 9, the burst sizes were relatively constant, but increasing the multiplicity of superinfection above 9 resulted in slight decreases in the burst size, which may have been due to lysis from without

TABLE 5

Effect of multiplicity of superinfection on recombination between first-step-transfer segment (FST) and two amber mutants am57 *and* am91

Cross	Multiplicity of superinfection	P.F.U. after superinfection	Progeny phages assayed on:		Burst size	Ratio of progeny phage assayed on W3110su⁻ ColIb⁻ to CR63su⁺ ColIb⁻ in per cent	
			CR63	W3110		Observed	Corrected
wild(FST) x am91	0	2.8×10^{7}	5.45×10^{9}	6.34×10^{9}	195	116	(100)
	2.3	1.81×10^{9}	3.64×10^{11}	3.17×10^{9}	201	0.87	0.75
	3.5	1.84×10^{9}	4.08×10^{11}	2.14×10^{9}	222	0.52	0.45
	7.0	1.68×10^{9}	2.99×10^{11}	7.70×10^{8}	178	0.26	0.22
	10.4	1.58×10^{9}	1.83×10^{11}	3.73×10^{8}	116	0.20	0.17
	13.9	1.89×10^{9}	9.80×10^{10}	1.50×10^{8}	52	0.15	0.13
wild(FST) x am57	0	3.5×10^{7}	7.10×10^{9}	7.29×10^{9}	203	103	(100)
	2.1	1.98×10^{9}	3.83×10^{11}	6.34×10^{10}	194	16.6	16.1
	3.2	2.01×10^{9}	4.75×10^{11}	5.32×10^{10}	237	11.2	10.9
	6.4	2.12×10^{9}	3.15×10^{11}	2.24×10^{10}	149	7.1	6.8
	9.5	1.90×10^{9}	2.00×10^{11}	1.17×10^{10}	105	5.9	5.8
	12.7	1.77×10^{9}	1.06×10^{11}	4.60×10^{9}	60	4.3	4.2

For the cross between wild-type FST segments and *am*91, the FST segment from wild-type phage was transferred, at a m.o.i. of 6.7, to CR63*su*+*Col*Ib⁻ cells, at 3.28×10^{9} per ml, by the procedure described in MATERIALS AND METHODS. The percentage of plaque-forming units (P.F.U.), of uninfected cells, and of adsorbed phage, before shearing the phage-bacterium complexes, was more than 99%, less than 1%, and more than 99%, respectively. After shearing, the percentage of colony formers was 4.7%. The efficiency of adsorption of the superinfecting *am*91, at the various multiplicities of infection indicated, averaged 99%. The cross between wild-type FST segments and *am*57 was carried out as with *am*91. The FST segment was introduced into CR63*su*+*Col*Ib⁻ at a m.o.i. of 6.1. The percentage of P.F.U., of uninfected cells, and of adsorbed phage, before shearing, was 120, 1.4, and 99%, respectively. After shearing, the percentage of colony formers was 4.7%. The efficiency of adsorption of the superinfecting *am*57 was 99%.

In all crosses, the multiplicity of superinfection is assumed to be the ratio of the total number of superinfecting phage to the total number of cells with which the cross was begun.

(Table 5). On the other hand, increasing the multiplicity of superinfection from any of the values reported in Table 5 resulted in considerable decreases in the yield of wild-type progeny phage assayed on W3110*su*⁻*Col*Ib⁻. These phenomena will be discussed later.

Superinfection with *am*91 yielded fewer wild-type progeny than the non-superinfected control at all the multiplicities of superinfection that were tested. However, superinfections with *am*57 yielded significantly more wild-type progeny than the control, at least when the multiplicity of superinfection was about 6 or less. It was not immediately obvious how to determine quantitatively this difference in the yield of wild-type progeny because of the effect of the multiplicity of superinfection. In order to assess this effect, the ratios of wild-type to total progeny phage from superinfections with *am*91 and *am*57 at various multiplicities were calculated (column 7, Table 5). These observed values were then

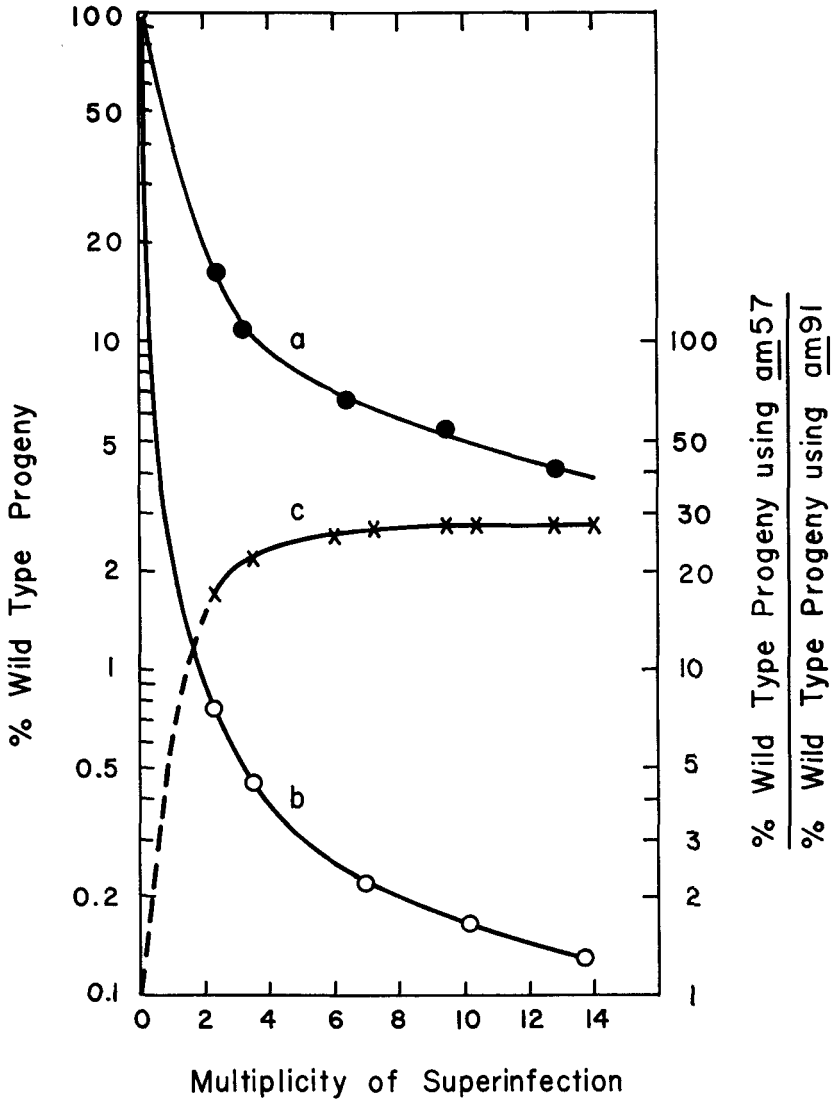

FIGURE 2.—Production of wild-type progeny phage in superinfected wild-type (FST)-cell complexes. The corrected values obtained in Table 5 were replotted as a function of multiplicity of superinfection. Curve a, wild type (FST) × *am57*; curve b, wild type (FST) × *am91*; curve c, ratio of the values of *am57* to that of *am91*.

corrected for plating efficiency on W3110*su*⁻*Col*Ib⁻ and CR63*su*⁺*Col*Ib⁻ (Table 5, last column) and plotted as a function of the multiplicity of superinfection (Figure 2, curves a and b). Again, it is seen that increasing the multiplicity of superinfection decreases the ratio of wild-type to total progeny formed regardless of the amber mutant used. However, if these ratios are themselves expressed as a ratio and plotted as a function of multiplicity of superinfection, it is seen that

increasing the multiplicity of superinfection above 6 does not change the ratio of wild-type progeny that results from superinfection by *am*57 to that resulting from superinfection by *am*91 (Figure 2, curve c). These data therefore demonstrate that *am*57 yields about 30 times more wild-type progeny than does *am*91 in this type of recombinational experiment.

Similar types of recombinational experiments were extended to *am*70, *am*218 (both defective in genes located in segment IV), and *h*1 mutants. The multiplicity of superinfection was fixed at about 6.0 to 7.5. The ratios of the number of wild-type to total progeny phage are shown in Table 6. When the FST segment originated from wild-type phage, both *am*57 and *h*1 yielded significantly more wild-type progeny (5.0–8.7%) than the other mutants (0.15–0.22%). When *am*57 or *h*1 was the source of the FST segment, superinfection by *h*1 or *am*57 respectively, gave significantly higher yields of wild-type progeny than superinfection by the other mutants.

From the data just described, we conclude that FST segments of BF23 can recombine with superinfecting phage genomes and that both the *am*57 and *h*1 mutations are located in the FST segment of BF23. Strong support for this conclusion is provided in the analysis of double-marker recombinants produced when various amber mutants superinfect cells harboring the FST segment from *h*1. In contrast to the production of wild-type progeny, *am*91, *am*70, and *am*218 yielded double-marker recombinants to approximately the same degree that *am*57 yielded wild-type progeny (Table 6).

DISCUSSION

Crosses between amber mutants representing 28 different cistrons were initially performed to provide a base for the determination of the relative map position of the mutation giving rise to the nonrestricted mutant, *h*1. The results of two- and three-factor crosses among these amber mutants may be summarized as follows. (1) Recombination frequencies among these mutants are very high with some crosses showing maximal values. 2) Recombination frequencies were additive up to 18–20%. 3) The genes identified in these experiments map into four segments; I (1), II (2), III (2), and IV (23), where the parentheses enclose the number of genes detected in each segment. 4) When the map distance between two genes is more than 100 units, the recombination frequency is maximal.

FATTIG and LANNI (1965) reported that 25 temperature-sensitive mutants of T5 represented 14 cistrons that comprised a single linkage group. However, some of the more recently isolated conditional-lethal mutants of T5 gave maximal recombinational value with some of the original 25 mutants, but nevertheless, could be located in the FST segment (LANNI, LANNI and TEVETHIA 1966; LANNI 1969). The recent isolation of an even larger number of conditional-lethal mutants of T5 has extended the T5 map to include four genetic segments (HENDRICKSON and MCCORQUODALE, in preparation), which are very similar to the segments of the genetic map of BF23 reported in this communication. The finding that current sets of mutants of both BF23 and T5 generate segmented maps, and

TABLE 6

Location of nonrestricted mutant, h1, *in first-step-transfer segment of BF23 chromosome*

Experiment	FST segment from:	Superinfected phage	Per cent of total progeny	
			h^+am^+ (wild)	h^-am^-
1	wild (h^+am^+)	$h1 \cdot am^+$	8.70	
		h^+am57	4.95*	
		h^+am91	0.22*	
		h^+am70	0.21	
		h^+am218	0.15	
2	$h1 \cdot am^+$	h^+am57	3.20	3.79
		h^+am91	0.15	7.37
		h^+am70	0.11	7.47
		h^+am218	0.13	6.81
3	h^+am57	$h1 \cdot am^+$	8.19	
		h^+am57	< 0.01	
		h^+am91	0.19	
		h^+am70	0.18	
		h^+am218	0.15	

The experimental procedure was essentially the same as that described in the legend of Table 5. FST segments were transferred at m.o.i.'s of 6.6, 5.9, and 6.8 to CR63*su*+*Col*Ib$^-$ at 3.44×10^9, 3.88×10^9, and 3.34×10^9 cells per ml in experiments 1,2, and 3, respectively. P.F.U., surviving colony formers, and efficiency of phage adsorption were all virtually identical to those of the experiments shown in Table 5. After shearing, the percentage of colony formers was 5.2, 4.4, and 6.0, and the multiplicities of superinfection were 7.4, 6.3, and 7.6 in experiments 1, 2, and 3 respectively. P.F.U. after superinfection varied from 50 to 70 percent of the number of cells with which the cross was begun. The values observed in experiments 1 and 2 were corrected for the efficiency of plating on W3110 compared to that on CR63, as shown in Table 5. The values of experiment 3 were not corrected in the foregoing manner since *am*57 does not plate on W3110.

* In other experiments, shown in Table 5, values of 6.20 and 0.22 can be interpolated from Figure 2 for a multiplicity of superinfection of 7.4 in crosses between h^+am^+ (FST) $\times h^+am57$ and h^+am^+ (FST) $\times h^+am91$, respectively.

the fact that both phages contain a single physical molecule of DNA, may be rationalized in two ways. First, if the 28 cistrons of BF23 are sufficient for constructing its genetic map, and if nicks are present in BF23 DNA that affect the recombination frequencies in some way, then the number of genetics segments may correspond to the number of physical DNA segments delineated by the nicks in the BF23 DNA. BUJARD (personal communication) has observed that the thermal melting profile of BF23 DNA has inflections virtually identical to those of the melting profile of T5 DNA, an indication that the structure of BF23

is the same as that of T5, which has a number of nicks in its mature DNA (ABELSON and THOMAS 1966; BUJARD 1969). A second explanation, less complicated than the first, is that the 28 cistrons detected are simply not enough to construct the genetic map of this phage. The first possibility, though attractive, seems unlikely, since the number of mutants assigned to each gene indicates that we are very far from a saturation level of mutations in BF23 as well as in T5. Furthermore, preliminary observations of recombination between BF23 and T5 amber mutants suggest that some T5 mutants show less than maximal recombination frequencies with both *am*57 and *am*63 or *am*111 of BF23, an indication that segments I and II are linked (ANDERSON and MCCORQUODALE, unpublished). If this be the case, the isolation of more mutants is required for the construction of the genetic map of BF23.

Two-factor crosses between the nonrestricted mutant *h*1 and various amber mutants failed to determine precisely the position of the *h*1 mutation relative to the amber mutations, but an observed recombination frequency of 21% (Table 2) suggested linkage with the *am*57 mutation. The location of the *h*1 mutation on the chromosome of BF23 could fortunately be assigned using a different approach, namely, recombination between FST segments and superinfecting phage genomes.

In establishing optimal conditions for recombination between FST segments and superinfecting phage genomes, we observed an effect of the multiplicity of superinfection on the yield of wild-type recombinants (Table 5 and Figure 2). As expected, superinfection of host cells harboring a wild-type FST segment by *am*57 (within the FST segment) yielded significantly more wild-type progeny than superinfection by *am*91 (not in the FST segment). However, in both cases, the yield of wild-type progeny decreased more rapidly than did the total burst size, as a function of the multiplicity of superinfection. These results suggested to us that a simple comparison of the number of wild type produced in the superinfected cultures with that in the nonsuperinfected cultures would not necessarily reflect the true recombination frequencies between genes in FST segments and genes in superinfecting phage genomes. However, by demonstrating that the ratio of the fraction of wild-type phage produced in cells superinfected with *am*57 to that produced in cells superinfected with *am*91 remained essentially constant as a function of the multiplicity of superinfection (Figure 2, curve c), we could conclude more confidently that about 30 times more wild-type progeny resulted from superinfection by *am*57 than from superinfection by *am*91. These data also led us to define a multiplicity of superinfection of slightly over 6 as one of our optimal conditions for this type of recombinational experiment.

When host cells that harbor a wild-type FST segment were superinfected with *h*1 or *am*57, about 25 to 40 times more wild-type progeny appeared than when *am*91, *am*70 or *am*218 was used as the superinfecting phage (Table 6, experiment 1). We interpret these results to mean that both *am*57 and *h*1 are located in the FST segment of BF23, and that both can recombine with wild-type FST segments. The best support for this interpretation is from crosses between FST

segments from either *am*57 or *h*1 and other mutants. When *am*57 was used as the source of the FST segment, only superinfections by *h*1 yielded wild-type progeny in numbers significantly larger than appropriate control superinfections (Table 6, experiment 3). Conversely, when *h*1 was used as the source of the FST segment, superinfection by all amber mutants tested, regardless of the map position of their defective genes, yielded double-marker recombinants (*h*1–*am*$^-$) at a frequency approximately equal to the yield of wild-type progeny resulting from superinfections by *am*57 (Table 6, experiment 2).

Crosses between FST segments (either from *h*1 or *am*57) and superinfecting phage genomes (either *am*57 or *h*1) yield lower frequencies of recombination between *am*57 and *h*1 than do normal crosses between these two mutants (Table 2). This difference may be explained by assuming that FST-DNA does not replicate and therefore cannot recombine repeatedly with the superinfecting phage DNA. Investigations of the physiological fate of FST-DNA under various conditions is now in progress.

It has been reported that the FST segment of T5 encodes the "pre-early" proteins (also referred to as "class I," or "very early" proteins; McCORQUODALE and BUCHANAN 1968) and that a mutation in gene I of the FST prevents the synthesis of one of these pre-early proteins (McCORQUODALE and LANNI 1970). Biochemical analysis of cells infected with *am*57 of BF23 has revealed that this mutant does not synthesize a pre-early protein that is functionally identical to the protein corresponding to gene I of T5. We have also observed that the mutation in *h*1 causes a change in the character of at least one pre-early protein, but no changes in early or late proteins, as judged by labeling patterns of electrophoretically separated proteins induced by the phage (MIZOBUCHI and McCORQUODALE, in preparation). These biochemical observations lend additional support to the genetic data presented in this paper.

The location of two genetic loci within the FST segment of BF23 discloses two features of the BF23 system. First the BF23 DNA is not permuted. If cyclic permutations of the genes did exist, crosses between the wild-type FST segment and any mutant would produce wild-type recombinants to the same degree. Second, the direction for injection of BF23 DNA is polarized, that is, segment I is always injected first. From the data of Table 6, however, we were unable to decide whether the genetic locus corresponding to *am*57 or *h*1 entered the host cell first. If we could identify a third genetic locus in the FST segment, which is linked with another segment, we could then determine the order that the FST genes are injected.

The nature of the mutation that produced our *h*1 mutant (and the other *h* mutants that we have isolated) is presently unknown since it was isolated as a spontaneous forward mutation from wild-type phage. It is important to determine whether the *h* mutants arise from point mutations or from multisite mutations, which may include deletions. However, it should be noted that regardless of the type of the *h*1 mutation, it occurs only in the FST segment, and thus the remaining 92% of the BF23 DNA does not direct the restriction mechanism by colicinogenic factor Ib.

Infection of *Col*Ib$^+$ cells by wild-type phage causes early abortive lysis of the host cells and restriction of phage growth. Mutation to *h* can overcome this early abortive lysis and consequently permits normal multiplication of BF23. The mechanism of this abortive lysis is not understood at the biochemical level but similar abortive systems such as T5 or T4 in Co270 (FIELDS 1969), T2 orT4 in ω-lysogenes (PIZER *et al.* 1968), and T4*rII* in λ-lysogens (SEKIGUICHI 1966) may provide us with some clues. It appears to us that further biochemical and genetic analysis of *h* mutants will provide a basis for understanding the interaction between colicinogenic factor Ib and BF23 DNA, and more generally, for explaining similar mechanisms of exclusion of a given phage or plasmid by another plasmid or phage genome harbored within host cells.

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SUMMARY

The growth of BF23, a bacteriophage related to T5, is restricted in cells colicinogenic for factor Ib. Mutants which can grow on such colicinogenic cells were isolated and one of them, *h*1, was characterized genetically in relation to a set of amber mutants isolated from wild-type BF23. Two- and three-factor crosses between the amber mutants, which represented 28 complementation groups, generated a genetic map consisting of four segments. Two-factor crosses between *h*1 and individual amber mutants failed to define the location of the *h*1 mutation relative to the amber mutations. However, crosses between just the 8% segment of the BF23 DNA that is initially transferred to host cells (the FST segment) and superinfecting phage genomes demonstrated that the genetic loci corresponding to *h*1 and to one of the amber mutants, *am*57, were both in the FST segment of BF23. The results indicate that BF23 DNA is not cyclically permuted and the direction of transfer of BF23 DNA to host cells is polarized, that is, the genetic segment containing the *h*1 and *am*57 loci is always transferred first.

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