Resistance to Colicins E3 and K Induced by Infection with Bacteriophage f1

(*E. coli*/gene-III product/membrane changes)

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ABSTRACT When infected with the filamentous phage f1, *Escherichia coli* K-12 strains are 20- to 40-times more resistant to colicins K and E3 than are uninfected cells. The colicins are adsorbed normally and are blocked in their action at some subsequent stage of their action. Productive infection of virions is not necessary for induction of resistance. The evidence presented implicates the product of gene III of the phage, a minor protein in the virion, as being responsible for this resistance. The simplest interpretation of the results is that this gene product blocks the penetration of colicins which is necessary for their action.

Among bacteriophage, the filamentous bacteriophage such as f1 (1) are unique in that they are released from the host bacteria by secretion through the cell membrane and wall rather than by cell lysis (2). Thus, infected cells continue to grow indefinitely, albeit at a somewhat reduced growth rate (dependent upon the conditions) and produce some 300 phage particles per bacterium per generation. During some experiments attempting to determine whether f1 infection was mutagenic, it was noted that f1-infected cells had become partially "immune" to treatment with either colicin K or E3. It is the purpose of this communication to document these findings and their implications with regard to colicin action.

MATERIALS AND METHODS

Bacteria were *Escherichia coli* K-12 strains K37 and K38 (3) and streptomycin-resistant derivatives: K140B (4), AB301 obtained from D. Morse, and W3110 and W3110 Col E3 obtained from D. Helinski. Strains K37 and K38 are intrinsically 20-times as resistant to colicins K and E3 as are AB301 and W3110.

Phage. Wild-type f1 (1) and a set of amber mutants were used (3, 4). The mutants are all derived from phage f1 except the one containing amber in gene VIII, which is derived from the closely related phage M13 and obtained from D. Pratt.

Colicins. Purified colicin K was obtained from M. Jesaitis. E3 was prepared from W3110 E3 (5). The final preparations generally had specific activities of about 10^6 killing units per μg of protein. One unit is defined as that amount of colicin which will reduce the titer of 10^8 bacteria (W3110) to 37% of its initial value.

Media. Phage and bacteria were grown in tryptone broth (3). Tryptone agar was used for phage plating.

Colicin Treatment. Logarithmic-phase cultures of bacteria in tryptone broth were mixed with an appropriate dilution of colicin. They were then either titrated for viable count after

20 min at ambient temperature and/or their optical density was followed as a function of time in a Gilford microsample spectrophotometer. 0.1 OD unit corresponds to a titer of 5×10^7 bacteria per ml with the measurements proportional to the viable titer in the range of 0.05-0.700 OD units.

RESULTS

Growth of Cells after Infection with Wild-Type Phage and Treatment with Colicin E3 or K. K38 was grown to 2×10^8 cells per ml at 37° ; half the culture was infected with a multiplicity of 50 plaque-forming units (PFU) of wild-type f1. This high a multiplicity insures the infection of 90% of the cells by 5 min after addition of phage. 15 min after infection the cultures were diluted 10-fold to fresh broth. 5-ml aliquots were distributed to 125-ml Ehrlenmeyer flasks and incubation with shaking continued to 45 min after infection. At this time various amounts of colicin K or E3 were added to the cultures. The OD at 700 nm was measured at 20-min intervals thereafter. Fig. 1 shows the growth curves obtained. With colicin E3, which stops protein biosynthesis by inactivation of ribosomes (6), at first the OD increases even though the cells are no longer viable (give a colony on plating). With colicin K, which stops all aspects of cellular metabolism (6), at sufficient concentration there is no further increase in the OD of the culture. In both instances we note that there is at least a 20-fold increase in the resistance of the infected cultures to treatment. The more sensitive indicator AB301 shows the same increase in resistance after infection, although the absolute doses used here were smaller, in keeping with this strain's greater colicin sensitivity (Fig. 2).

The difference in the initial OD for the infected and uninfected samples is due to the fact that f1-infected cells grow at only three-fourths of the rate of uninfected cells. Thus 45 min after infection, the two cultures differ by about a factor of two in cell number. Therefore, in the experiments described above, the colicin concentration per cell was 2-fold higher for the infected sets. Despite this fact, the resistance of the infected cells is still very apparent.

In those instances where the growth rate is unaffected by the colicin, there is close correlation between the OD and the viable cell number as measured by ability to form colonies. In those instances in which the growth rate, as measured by OD is affected, however, the OD overestimates the number of viable cells. Apparently some cells can grow for a bit but are ultimately destined to die.

Colicin Receptors. One possible explanation for the f1-induced resistance was a partial loss of colicin receptors. However, colicins K and E3 have different receptors on the cell

surface; those of the former correlate with those for coliphage T6 and the latter with coliphage BF23 (7). Still, to explicitly test this possibility, I measured the adsorption of colicin. A culture of K38 was infected with f1 for 45 min and an uninfected culture was set to the same cell number. Colicin K or E3 was added to each culture at 10 killing units per ml. In addition, tubes containing only the colicins and no bacteria were subjected to the same manipulations. After incubation for 10

min at 37° the cultures were centrifuged at $30,000 \times g$ to remove the bacteria. The supernatants were added to the K38 SM^r bacteria, and after 15 min of incubation were titrated for bacterial survivors on plates containing streptomycin. This procedure allows for scoring of the colicin sensitivity of the tester bacteria since the centrifugation step does not remove all of the original bacteria (K38).

Since the tester cells survive in the supernatants from both infected and uninfected cells (Table 1), the colicin has been removed. We can assume that there has been no gross change in ability of infected cells to adsorb colicin. It is possible that

FIG. 1. Resistance of cultures of K38 to colicin treatment 45 min after infection. *Solid lines* are for uninfected cultures; *dashed lines* are for infected cultures. μg of colicin per ml of culture: (A) Colicin E3: (○) 0, (Δ) 0.06, (●) 0.6, (□) 6.0. (B) Colicin K: (○) 0, (Δ) 0.1, (●) 1.0, (□) 10.0.

FIG. 2. Same as legend for Fig. 1 but culture used was the more sensitive one, AB301. μg of colicin per ml of culture: (○) 0, (Δ) 0.01, (●) 0.1, (□) 1. (A) Colicin E3; (B) colicin K.

TABLE 1. Adsorption of colicins K and E3 by f1-infected cells

	Infected		Uninfected		Controls	
	K	E3	K	E3	K	E3
Bacteriophage	1.2×10^8	1.3×10^8	1.4×10^8	1.2×10^8	2×10^4	8.3×10^3

Colicin-treated f1-infected cultures, uninfected cultures, and tubes containing colicin alone were centrifuged after 10 min of incubation to remove bacteria. Streptomycin-resistant bacteria were added to give 1×10^8 bacteria per ml. After 15 min, the cultures were plated on plates containing streptomycin (100 μ g/ml). After overnight incubation, colonies were counted.

f1 infected cells will adsorb fewer total colicin molecules but because of the imprecision in the colicin assay, saturation experiments were not attempted.

Another type of experiment illustrates the same point. A phage mutant was used that induces colicin resistance to an even greater extent than does wild-type f1. The cells were infected and incubated for 30 min at 37°. An aliquot was challenged with phage T6 before or after the addition of large amounts of colicin K or E3. Table 2 shows: (i) that f1-infected cells are sensitive to T6 and (ii) when colicin K but not E3 was added before challenge by phage T6, the cells were protected from T6. Apparently the T6 receptors had been "covered" by colicin K but not by colicin E3.

Genetic Control of Resistance to Colicin. For determination of whether a specific phage gene product was involved in induction of colicin resistance, rather than being the result of a generalized effect of either infection or phage production, two types of experiments were done.

The first set of experiments took advantage of the fact that f1 undergoes a restriction-modification cycle (8) when grown on *E. coli* with B or K specificity. Wild-type phage and various mutants partially or fully resistant to B restriction were tested for their effect on the colicin K resistance of B-type infected cells. All of the phage strains induce equal resistance when they infect K cells. Table 3 shows that there is a pronounced correlation between the relative resistance induced and the sensitivity to restriction. The higher values for restriction sensitivity relative to colicin sensitivity are probably due to the

higher multiplicity of infection. High multiplicity of infection causes an increased escape from restriction for many phage (9), and also for f1 (10). This experiment indicates that the resistance induced is proportional to infectivity of the phage and, therefore, that some phage gene product is involved.

This particular point was verified in the second set of experiments which were designed to determine whether there was a particular gene product involved. The experiment was complicated by the fact that although wild-type f1 doesn't kill infected cells, all conditional lethal mutants, except those in gene II (11), kill the cells under nonpermissive conditions. However, the gene-II phenotype in this respect is dominant; all double mutants of the type gene II and gene "X" do not kill under nonpermissive conditions (12). Gene-II mutants block phage DNA replication beyond the conversion of the input single-stranded genome to the double-stranded replicative form (12). Thus when a gene-II mutant is used, sufficient gene products must be made from input replicative forms for any protection to be observed. Studies of two phage gene products, those of gene VIII (13) and gene V (14), have shown that considerable, although less than normal, amounts are made under such conditions. Preliminary experiments with gene-II mutants showed that 20 min after infection—before the replicative forms begin to segregate due to cell division—they conferred a level of colicin resistance comparable to that induced by wild-type phage infection. This result in itself shows that production of phage particles is not a necessary precondition for induction of colicin resistance.

Since the recombination frequency of f1 is very low (3), it was necessary to obtain the requisite double mutants by iterated mutation. At least one representative mutant in each of the eight from genes of f1 was coupled to a gene-II amber by mutagenesis (3). The genotype of each double mutant was verified by complementation tests with tester stocks (3).

Table 4 lists the relative resistance to colicin K, 20 min after infection with high multiplicities of the various mutants. A similar, less complete set, of data is also available for colicin E3. For amber mutants: phage with mutations in genes

TABLE 2. Effect of phage T6 challenge on phage R113-infected cells

	Uninfected	Infected
E3	$<10^{-5}$	1
K	$<10^{-5}$	1
E3 then T6	$<10^{-5}$	1.1×10^{-2}
K then T6	$<10^{-5}$	1
T6	6×10^{-3}	9×10^{-3}

A logarithmic-phase culture of K37 was grown to 1.5×10^8 cells/ml in tryptone broth. Half the culture was infected with phage R113 (see Table 4). Both cultures were diluted so that at 15 min after infection there would be 1.5×10^8 cells per ml. The cultures were treated with colicins E3 and K as described in Table 4 with 15 killing units each. Phage T6 (multiplicity of infection = 5) was added when appropriate 10 min after addition of colicin. 10 min later the cultures were diluted and plated for viable cells. Note that K37 cultures contain about 10^{-5} cells that produce mucoid colonies that are "resistant" to most phage and colicin. Numbers in this table are relative to total cell number, at the time after addition of T6.

TABLE 3. Effect of restriction of phage f1 on colicin K protection

Phage	Plating efficiency ^a	Relative protection ^b
f1(B)	1	1
f1SB ₁ [°] SB ₂ [°] (K)	1	1
f1SB ₁ [°] (K)	3×10^{-2}	9×10^{-2}
f1SB ₂ [°] (K)	3×10^{-2}	5×10^{-2}
f1(K)	8×10^{-4}	1.5×10^{-3}

^a Low multiplicity. ^b High multiplicity.

I, IV, VII, and VIII and necessarily gene II confer resistance on the cells equal to that conferred by wild-type phage at 20 min after infection. Phage with mutations in gene V and VI are slightly less effective but confer considerable resistance compared to uninfected cells. The reasons for this lower efficiency are unknown, but since only one mutation in these genes has been tested, this could be the result of other unknown incidental mutations in the stocks. Alternatively, the genes themselves may be more directly involved in an unknown manner. For example, gene V specifies a DNA-binding protein (15) whose absence might affect the transcription of replicative form and hence the amounts of gene products.

Only mutants in gene III show significant effects. Among the ones studied, paradoxically, every possible effect is found. Thus, three different amber mutants do not protect at all, one amber mutant gives normal protection, and the one temperature-sensitive mutant protects better than wild type both at the permissive and nonpermissive temperature. This mutant confers about the same level of resistance to the sensitive indicator AB301 as to the more resistant K38. Reversion of this lesion returns this phage stock to the protection level afforded by wild-type phage. Crosses between these mutants show them to be at different sites, but because of the manner in which the crosses must be done (3), their relative positions in the gene are uncertain.

DISCUSSION

The experiments described here demonstrate that cells infected with phage f1 are resistant to various colicins. Smilowitz (*J. Virol.*, submitted) has made similar observations. Colicin K and E3 have been studied extensively, and preliminary results indicate that the same is true for colicin E2. Importantly, K and E3 have different receptors on the outer membrane and direct experiments show that the resistance is not due to a loss of receptors. Thus, f1-infected cells resemble colicin-tolerant mutants (7, 16), particularly those of the tol-II group which confer partial resistance to various colicins. These mutants also have profound effects on the cells' inner membrane (7). f1 infection can produce significant changes in cell membranes (17).

Several lines of evidence point to a unique phage specific product for induction of colicin resistance. Infection itself is insufficient, and production of viable phage is not necessary. When phage infects a restricting host the resistance is proportional to the degree of restriction. Mutants in gene II, which prevent phage DNA replication, still confer resistance. Apparently the replicative form derived from the input phage can direct the production of a considerable amount of gene products, as had been suggested (13, 14).

Which gene product is involved is still questionable, but the evidence points to that of gene III. Gene-III product is the largest of the phage-specified proteins (70,000 molecular weight) and is a minor protein in the virion itself (18, 19). The difficulty in being certain is due to the fact that gene III appears to be the first gene in an operon containing, in this order, genes VI and gene I (11). Mutants in these latter two genes protect and hence are not involved. Therefore, either there is another unknown gene between III and VI or we must conclude that the fragment produced by III₈₇ can still protect, since both III₈₇ and III₁₁₇ are nonpolar. Gene III is further implicated by the fact that the *ts*III mutant studied, protects to an even greater extent than does wild-type phage.

TABLE 4. *Relative resistance of various mutant infected cultures to colicin K*

Strain	Gene ^a	Comments	Relative to wild type ^b
R22	AmI ₃		1
R118	AmI ₁₁₈		1
R135	II ₁₃₅		1
R28	AmIII ₄	Polar on VI and I	<10 ⁻⁴
R89	AmIII ₈₉	Polar	<10 ⁻⁴
R117	AmIII ₁₁₇	Nonpolar	<10 ⁻⁴
R87	AmIII ₈₇	Nonpolar	1
R90	AmIV ₉₀		1
R96	AmIV ₉₆		1
R30	AmV ₁₃		0.2
R27	AmVI ₅		0.3
R152	AmVII ₁₅₂		1
R153	AmVIII ₁₅₃		1
R104	<i>ts</i> I		0.3
R113	<i>ts</i> III		5-10
R145	<i>ts</i> IV		1
R101	<i>ts</i> V		1

^a In addition to an amber mutant in gene II.

^b Logarithmic-phase K38 cells were infected with a multiplicity of infection = 50 of the different phage strains. Colicin K was added to 10 killing units per ml. The reaction was stopped by 1/100 dilution at 20 min. The experiments were done at least twice for each mutant and always with control of wild-type infected and uninfected cells. Different cultures had different sensitivities and induced resistance. Thus, for wild-type infected cells the number of survivors varies between 10 and 20% of cells. The symbol 1 refers to a culture being within 30% of the value of the control culture for that particular experiment. For the *ts* mutants, the nonpermissive temperature was 42°.

How the protection occurs is obscure. An analogy might be drawn between phage penetration and the necessity for colicins to enter cells to produce their effects (21-24). Phage gene-III product is the attachment protein of this phage (12) and is probably involved with its penetration (19, 20) and perhaps with its egress.

There is a kind of stoichiometry in the resistance induced in different hosts. Thus, the relative degree of protection afforded by infection is the same regardless of whether the host was a sensitive or partially resistant one. It is as if normally a colicin molecule had some probability of killing each kind of cell and this was reduced by a constant fraction. This might be accomplished by direct blockade by gene III or other protein, should the phage and the colicins use the same mechanism of entry and egress. To draw the analogy still closer between colicin penetration and f1 penetration, Smilowitz (*J. Virol.*, submitted) has shown that phage f1 does not penetrate col tol-II mutant cells although it does attach to them.

Colicin E3 can also be assayed by its inhibition of protein synthesis *in vitro* (21, 23). In addition, an immunity substance specified by this Col factor can prevent E3 action (21, 22, 23). However, all attempts to mimic the action of the immunity substance by addition of extracts from infected cells have failed. Perhaps it is necessary to have purified gene-III protein.

The colicin resistance produced by phage-f1 infection adds to our list the known effects of phage infection upon bacteria and, by inference, of viral infection on cells (25). Some phages,

such as the T-even set, completely subvert the host metabolism to their own growth. Other, more temperate, phages can be integrated into the genome of the cell and produce "lysogenic conversions or mutations" (25, 26) of cell properties. In these latter instances replication of phage DNA is synchronous with that of the host and most of the phage's genes are repressed. With phage f1, on the other hand, the cells continue to grow with at least two new genetic phenotypes, the extrusion of the nucleoprotein particles we call the virion f1, and a partial resistance to colicin.

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