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Colicin Activity and Abortive Infection of T5 Bacteriophage in *Escherichia coli* (ColIb)

DONNA H. DUCKWORTH,* GLENDA B. DUNN, THOMAS PINKERTON, KATHERINE ROSE, AND SATISH K. WALIA

Department of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, Florida 32610

We performed three types of experiments to test the hypothesis that abortive infection of T5 bacteriophage in *Escherichia coli* (ColIb$^+$) is due to internally released colicin. (i) We measured the sensitivity of cells to colicin under a variety of conditions and then looked at the plating efficiency of T5 in ColIb$^+$ cells under these same conditions. Cells grown at 42°C or with hexanol had a reduced sensitivity to externally added colicin and an increased efficiency for T5 when the ColIb plasmid was present in the infected cells. Phage growth was far from normal, however. (ii) We measured the colicin sensitivity of a mutant bacterium that grew T5 normally even in the presence of the ColIb plasmid and measured the plating efficiency of T5 on another mutant that was colicin tolerant. Here again, the correlation between colicin activity and inhibition of phage replication was not complete. (iii) We looked for colicin-negative plasmid mutants and tested the ability of cells containing these plasmids to support the growth of T5. These experiments used Tn*5*, a kanamycin resistance transposon, as the mutagen. All possible combinations of colicin production and phage inhibition were found, including mutants that produced no colicin but still inhibited phage production.

T5 and BF23 are two phages that share many characteristics, including some that are virtually unique (16). Besides having a distinct type of DNA (14, 16, 20), these phages have a unique two-step injection mechanism for their DNA. They are also unusual in that they will not grow in bacteria containing the colicinogenic plasmid ColIb (19, 22). The inhibition of phage growth is not due to an inability of the phage to adsorb or inject their DNA, and the DNA is not broken down, as it would be if restriction enzymes were preventing the phage replication. Instead, the infectious cycle of the phage proceeds through the pre-early phase and then stops abruptly (5, 16). In this respect, the abortive infection of T5 in the presence of the ColIb plasmid is similar to the inhibition of T7 caused by the F (fertility) factor (4, 18) and the exclusion of T4rII mutants by lysogenized λ phage (21). Based on the expectation that the ColIb-induced abortive infection would be similar to these other abortive infections and involve changes in the cell membrane, we have looked at membrane function during T5 infections of ColIb-containing cells.

We have found that dramatic changes in the functioning of the cell membrane do, indeed, occur. These include inhibition of proline, glutamine, leucine, and thiomethylgalactoside transport, stimulation of α-methylglucoside uptake (3, 5), increased fluorescence intensity of membrane-bound *N*-phenyl-1-naphthylamine (6), and release of intracellular K^+ and Mg^{2+} (7). Because the ion depletion occurs before the cessation of macromolecular syntheses during the abortive infection, we have concluded that membrane depolarization due to ion pore formation is, in fact, the cause of the abortive infection. This conclusion is substantiated by the fact that mutants of either the phage or plasmid that prevents the abortive infection also prevent the membrane changes.

Because the membrane changes which occur during the abortive infection also occur when the colicin Ib protein acts to kill sensitive cells, McCorquodale has hypothesized that it is the colicin protein itself that is the cause of the abortive infection (17). He proposes that the phage may inactivate the colicin immunity protein, leading to membrane depolarization and cell death caused by the colicin. In an attempt to prove or disprove this hypothesis, we performed three types of experiments: (i) we tried to find conditions which would prevent colicin killing of cells and then determined whether ColIb$^+$ cells grown under these same conditions allowed T5 to grow; (ii) we measured the sensitivity to colicin of mutant bacteria that could grow T5 normally even in the presence of the ColIb plasmid and measured the growth of T5 in the presence of the plasmid in another *Esch-*

erichia coli mutant that was tolerant to the killing action of colicin Ib; and (iii) we looked for colicin-negative plasmid mutants and tested the ability of cells containing these plasmids to support the growth of T5. The results were, we feel, tantalizingly suggestive but not conclusive.

MATERIALS AND METHODS

Organisms and media. Our bacterial strains, pertinent characteristics, and sources are listed in Table 1. T5 and BF23 phages were obtained from R. Benzinger, and λ:Tn*5* was obtained from M. Malamy. High-titer stocks were obtained by the confluent lysis method of Adams (1). Phage titers were determined by the agar layer method (1). The medium used was H broth (8 g of nutrient broth, 5 g of peptone, and 5 g of NaCl per liter) or H agar. For the experiments using hexanol, 1/1,000 volume of this was added to either the liquid medium or the agar for the plates. For the growth of λ and infection of cells with this phage, maltose was added to the medium.

Colicin assays. To check for the production of any colicin, 1- or 2-day-old sparsely seeded plates of the bacteria to be tested were overlaid with a lawn of the indicator strain, K12S, in soft agar and incubated for an additional 8 to 18 h. Colicin producers showed a clear zone of killing around the colonies. Flooding the plates with $CHCl_3$, followed by removing $CHCl_3$ and evaporating any residual $CHCl_3$ before the overlay of K12S, caused the formation of larger and sharper killing zones, but did not in any case change the results from negative to positive. Hence, flooding was not always done. To quantitatively measure colicin killing, the supernatant fluid from a 1- or 2-day-old culture of a colicin producer grown in liquid medium was removed by centrifugation and sterilized by one or two passages through a membrane filter (Millipore Corp.). The sensitive (test) cells were grown in H broth to 5×10^8 cells/ml; they were then centrifuged and suspended at the same cell density in a minimal medium with no carbon source. Colicin was added (typically to 2×10^8 cells), and the cells were incubated at 37°C for 1 h. After this, the cells were diluted, plated on nutrient agar plates, and incubated overnight to determine the number of viable cells. The percentage of survival is calculated by comparing the number of cells in the colicin-treated culture with the number of cells in an untreated sample.

Selection of antibiotic- or phage-resistant organisms. The antibiotics used were all purchased commercially. They were used in the following concentrations: rifampin, 200 μg/ml; kanamycin, 50 μg/ml; tetracycline, 25 μg/ml; and nalidixic acid, 30 μg/ml. The rifampin-resistant mutants of JK115 and RM42 were selected by plating about 10^7 cells (not mutagenized) on agar plates containing 200 μg of rifampin per ml. Colonies which grew were restreaked on rifampin-containing agar and checked for the appropriate phenotype (phage growth, colicin sensitivity, sugar metabolism). The Nal^r RM43 was obtained by the same procedure except that nalidixic acid was present in the plates instead of rifampin. To obtain RM42 Rif^r/λ^r (lambda resistant) cells, 10^8 RM42 Rif^r cells were plated with about 10^6 λ phage and incubated overnight. From the confluently lysed plate, colonies were picked and tested for phage λ growth, T5 growth, rifampin resistance, and lactose fermentation.

To obtain the ColIb-containing JK115 cells, JK115 Rif^r was mated with RM43 and streaked onto rifampin-containing agar. Individual colonies were then screened for the production of colicin. RM43(KColIb)

TABLE 1. *Strains of bacteria used*

Strain designation	Relevant genotype or phenotype	Source	Reference
B	*E. coli* B, prototrophic	M. J. Bessman	
RM42	*E. coli* W3110 *thy* $ColI^{r a}$	R. Moyer	5
RM43	= RM42(ColIb-P9)	R. Moyer	5
UA489	*E. coli* K-12 F^-, multiply auxotrophic	E. E. Moody	8
UA490	= UA489 *cmrA2 cmrB2*[b]	E. E. Moody	8
UA491	= UA489(ColIb-P9)	E. E. Moody	8
UA492	= UA490(ColIb-P9)	E. E. Moody	8
JK115	*E. coli* K-12 DG 78-X36F^- *tolI*,[c] multiply auxotrophic	D. J. McCorquodale	2
FK33	*E. coli* K-12 F^- (pFK33)[d]	M. Malamy	
K-12S	*E. coli* K12 ERL[e] #22R80, sensitive to all colicins	K. B. Sharma	Walia[f]
BM21	*E. coli* K-12 F^- Nal^r	K. B. Sharma	Walia
BM21(KColIb)[g]	*E. coli* K-12 F^- Nal^r (K ColIb)	K. B. Sharma	Walia
SW9-4–SW10-32	= BM21(ColIb:Tn*5*)	This laboratory	This paper
GD4–GD128	= RM42(ColIb:Tn*5*)	This laboratory	This paper

[a] Lacking the cell surface receptor for colicin Ib.
[b] Permissive for T5 in $ColIb^+$ cells.
[c] Adsorbs colicin I but is not killed by it.
[d] F factor *Eco*RI fragment 7 carrying resistance to tetracycline and kanamycin.
[e] Enteric Reference Laboratory, Colindale, England.
[f] S. K. Walia, Ph.D. thesis, Maharshi Dayanand University, Rohtak, India, 1979.
[g] Plasmid $TP112^{drp}$ carrying kanamycin resistance and colicin Ib activity, originally isolated from *Salmonella typhimurium*.

was obtained by mating RM42 Rif^r with BM21(KColIb) and selecting for colonies on rifampin-kanamycin agar. These were then checked for colicin production.

Conjugations. Cells were grown in H broth for 10 to 12 h at 37°C with minimal shaking. Donor and recipient cells were mixed in equal amounts and incubated with no shaking at 37°C for 2 h. Then 0.1 ml of the mixture was plated on the appropriate selective medium. If the donor was a colicin producer, the cells were centrifuged, washed, and suspended in fresh broth before mixing.

For the triparental cross involving FK33, RM43, and JK115, late-log-phase cultures of FK33 and RM43 were centrifuged, washed, suspended, mixed in equal amounts, and incubated for 2 h before the addition of JK115. This culture was incubated for 2 h more or overnight.

RESULTS

Alteration of the cell membrane. Since the colicin Ib protein is a membrane-active colicin (17), we tried growing cells under conditions that would alter their membranes to see if this would change their sensitivity to colicin. We initially tried growing the cells at 42°C, one condition known to alter cell membranes (15). This treatment somewhat decreased the sensitivity of the cells to colicin (Table 2) and increased the number of plaques formed on RM43 by about 10-fold. However, the plating efficiency remained far from normal.

Since cells grown at high temperatures alter their lipid composition to compensate for the increased membrane fluidity (13, 15), we investigated whether cells grown in 0.1% hexanol (which also causes altered lipid composition [9, 10]) would, perhaps, be not susceptible to killing by colicin Ib. Here again, the killing ability of the colicin was reduced and there was an increase in the plating efficiency of T5 on RM43, but again the plating efficiency was still very low compared with that of cells without the ColIb plasmid (see Table 2).

Studies with mutant bacteria. In 1976, Hull and Moody (8) reported that they had isolated a mutant *E. coli* that would grow T5 normally, even when the ColIb plasmid was present in the cell and producing colicin normally. The mutation was found to map near the genes for streptomycin resistance and some ribosomal proteins, but the phenotypic change preventing the abortive infection was not identified. One possibility, suggested to us by D. J. McCorquodale, was that the mutant had become tolerant to the effect of colicin Ib and hence unable to undergo an abortive infection. To test this, we used strain UA490, which contains the mutation but not the plasmid, and looked for killing by colicin Ib. We found, in several tests,

TABLE 2. *Correlation between colicin sensitivity and plating efficiency of T5*

Culture	% Survival after colicin[a]	% Plating efficiency of T5
E. coli B at 37°C	23, 28, 53	100
E. coli B at 42°C	41	100
RM42 at 37 or 42°C	—[b]	100
RM43 at 37°C	—	10^{-5}
RM43 at 42°C	—	10^{-4}
E. coli B + hexanol at 37°C	66, 68	100
RM42	—	100
RM42 + hexanol	—	100
RM43	—	10^{-5}
RM43 + hexanol	—	10^{-4}–10^{-2}[c]

[a] Cells were incubated for 1 h with colicin and then plated for determination of colony formers.

[b] Could not be measured because RM42 is resistant to externally added colicin.

[c] 10^{-2} represents very tiny, barely visible plaques.

that UA490 could be killed by colicin to the same extent as the control (*E. coli* B or UA489); it also could grow phage normally even when the ColIb plasmid was introduced into it, as has been reported (8). Hence, the lesion allowing phage growth was not colicin tolerance.

In contrast to this mutant, strain JK115, which was isolated as a strain having receptors for colicin Ib but not affected by the killing action of the protein (2), was totally immune to the effects of the colicin. Because this was a truly tolerant strain, we decided to introduce the ColIb plasmid into it and see if T5 could grow. If the colicin protein was causing the abortive infection, this strain might grow T5 normally even in the presence of the plasmid. A number of isolates of JK115(ColIb$^+$) were tested for their ability to grow phage BF23. This phage was used because we found that JK115 cannot adsorb T5. Revertants of one of the isolates, as well as acridine orange-treated cells which retained the plasmid, were all tested for phage growth (Table 3). In summary, we can say that when JK115 contained the ColIb plasmid, plating efficiency was on the order of 1% of normal. When JK115 did not contain the plasmid, plating efficiency was normal. Growth of JK115(ColIb$^+$) in hexanol or at 42°C slightly increased the plating efficiency of phage (two- to threefold) but did not increase it to 100%. We had thought that the tolerance mutation and membrane alteration might act synergistically to give normal phage growth. Rather, it appeared that the change in JK115 might be the same as or similar to that caused by hexanol or 42°C, so that these treatments had little effect. When the K ColIb plasmid was introduced into JK115, phage produc-

TABLE 3. *Plating efficiencies of BF23 on some colicin-tolerant strains*

Strain	% Plating efficiency
JK115	100
JK115 Rif^r	96–118
DWXXIV [JK115 Rif^r (ColIb$^+$)]	0.8–3.1
DWVIII [JK115 Rif^r (K ColIb$^+$)]	1.2–12
DWXXIV rev[a] [JK115 Rif^r]	96
DWXXIV [JK115 Rif^r (Col Ib)][a]	1.5
RM42	100
RM43	2×10^{-4}
RM42(KColIb)	2×10^{-2}

[a] Treated with acridine orange.

tion was as high as 12% of normal. However, this plasmid had a much reduced ability to inhibit phage growth when introduced into RM42. Interestingly, the K ColIb plasmid produced as much colicin as did the ColIb plasmid. Conceivably, its immunity system is less easily inactivated, however. So it does appear that the mutation to colicin tolerance allows the phage that are normally inhibited by the ColIb plasmid to grow at a higher level than in strain RM43. Whether this increased growth is due to the tolerance mutation or another undetected mutation or even the genetic background in JK115 is not known.

Plasmid mutants. We have begun experiments to isolate and characterize specific mutants of the ColIb plasmid: mutants that are colicin negative or that allow phage replication. To see whether synthesis of colicin is an absolute requirement for phage inhibition, colicin-negative mutants were tested for phage growth, whereas those that allow phage growth were tested for colicin production. Very few reports on ColIb plasmid mutants exist. R. Moyer isolated a plasmid mutant that allows normal phage growth (5). We found this mutant to produce normal amounts of colicin. Several colicin-negative plasmid mutants were studied by Hull and Moody (8), and they reported that many allowed phage replication. Because none of these mutants was shown to be mutant at a single site and hence could contain multiple mutations, we decided to use Tn*5*, a transposon coding for kanamycin resistance (12), as a mutagen. Since analysis of the DNA from plasmids mutated by the insertion of Tn*5* will allow determination of the site of mutation, these studies should be more conclusive.

As a source of Tn*5* we have used the pFK33: Tn*5* mini-F plasmid of M. Malamy. In these experiments, a triparental mating of FK33, RM43, and JK115 Rif^r was done. The ColIb plasmid presumably goes initially into strain FK33, where it can pick up the transposon and then transfer itself to JK115. JK115, a colicin-tolerant strain, was used here to obviate any possible killing by RM43. Transconjugants were selected for resistance to rifampin and kanamycin and sensitivity to tetracycline. Since pFK33 carries a gene for tetracycline resistance, as well as the kanamycin-resistant Tn*5*, these cells would be those to which Tn*5*, but not pFK33, had been transferred. Presumably, Tn*5* could only have been transferred if it were inserted into the ColIb plasmid. These cells would, therefore, be JK115 Rif^r (ColIb:Tn*5*).

From these Rif^r Kan^r colonies, colicin-positive or -negative cells were picked. To ensure that kanamycin resistance was truly plasmid associated in these cells, a further transfer to BM21 Nal^r was done. Transconjugants from these matings that were Nal^r Kan^r were then selected and rescored for colicin and phage production (Table 4). Although some colicin-negative transconjugants were, indeed, permissive for phage growth, others were still non-permissive. Also of note is the mutant SW9-13, which produced colicin and was permissive. A mutant of this type was previously isolated by R. Moyer, however (5).

ColIb:Tn*5* plasmids with altered phenotypic properties were also obtained by infection of RM43 with λ:Tn*5* followed by conjugation with RM42 Rif^r λ^r. Rif^r Kan^r colonies were then selected and tested for their ability to transfer kanamycin resistance, inhibit phage replication, and produce colicin. Representative colonies

TABLE 4. *Colicin production and phage production in E. coli containing Tn5 insertion mutants of the ColIb plasmid*

Strain	Colicin production[a]	Phage production[b]
SW9-4	+	−
SW9-13	+	+
SW9-23	+	−
SW9-39	+	−
SW10-1	−	−
SW10-8	+	−
SW10-13	−	−
SW10-25	−	+
SW10-30	−	−
SW10-32	−	+
GD4	−	+
GD9	−	+
GD26	+	−
GD29	+	+
GD30	+	−
GD87	+	+
GD122	+	+
GD128	+	+

[a] Measured by a zone of killing around 1- or 2-day-old colonies overlaid with K-12S.

[b] Determined by plating at least 300 T5 phage per plate with the indicated strain.

from these experiments are also listed in Table 4. These (GD4 through GD128) differed from the SW mutants in that they were in the RM42 Rif^r rather than the BM21 Nal^r background. Predominant among this group were colicin producers that allowed phage production.

We are in the process of isolating plasmid DNA from all of these strains for restriction enzyme analysis. With strains that are colicin negative and allow phage growth, we will be looking especially for multiple insertions of Tn*5* or for large deletions of plasmid DNA. It is even possible that these strains may have completely lost their plasmid. Although final conclusions cannot be reached until studies on the DNA are complete, phenotypic analysis of the Tn*5*-containing mutants does not offer strong support for the hypothesis of abortive infection caused by the colicin protein. As explained in Discussion, though, the results by no means disprove the hypothesis.

DISCUSSION

When *E. coli* containing the ColIb plasmid is infected by T*5*, the early stages of the phage replicative cycle proceed normally (16). At 6 to 10 min postinfection, however, a cataclysmic event occurs which causes all macromolecular synthesis to cease and precludes the production of any progeny phage (5). We have found that cell membrane depolarization precedes the cessation of macromolecular synthesis and have hypothesized that this leads directly to the abortive infection of the phage (6, 7). Because cell membrane depolarization also occurs when sensitive cells are attacked by the colicin Ib protein, it has been hypothesized that T5 phage infection inactivates the colicin-producing cells' immunity system, allowing intracellular colicin to "kill" the infected cells (17). The experiments reported in this paper were done to either prove or disprove this hypothesis.

In the first group of experiments, we tried to alter the membranes of cells to such an extent that they would no longer be sensitive to the colicin protein. We did this by growing the cells at an elevated temperature and in the presence of hexanol, two conditions known to alter membrane fluidity (9, 10, 13, 15), and, consequently, the protein composition of the cell membrane (11; L. O. Ingram, personal communication). We found that both of these conditions caused the cells to be less sensitive to colicin, and both caused an increase in plating efficiency of T5. In neither case was the effect dramatic, however. The colicin sensitivity was reduced by less than 2-fold, and although the plating efficiency of T5 was increased 10-fold, or even more if one counts the tiny, cloudy plaques that were barely visible, it remained very low compared with that of cells with no plasmid. Still, it could be reasonably concluded from these results that the colicin was causing the inhibition of phage replication in ColIb$^+$ and that there was enough intracellular colicin released to inhibit even the less sensitive (altered membrane) cells. Alternatively, the change in the cell membrane induced by the high temperature or hexanol may have inhibited the interaction with a separate phage inhibition protein in the same way that the interaction with the colicin protein was inhibited.

Next we looked for two bacterial mutants that affected either the plating efficiency of T5 in the presence of the ColIb plasmid or the sensitivity of the bacteria to colicin. In 1976, Hull and Moody (8) reported the isolation of bacterial chromosomal mutants that would grow T5 normally, even when the ColIb plasmid was present in the cells and producing normal amounts of colicin. We investigated whether this bacterial mutant was, perhaps, tolerant to the killing action of colicin and found that it was killed to precisely the same extent as *E. coli* B or its parent strain, UA489. From this we can say that either the colicin protein was not causing the abortive infection or the mutation in strain UA490 was preventing the abortive infection in an unknown manner. Studies with JK115, a truly colicin-tolerant mutant (2), appeared to be more suggestive. We introduced the ColIb plasmid into strain JK115 and investigated whether BF23 would infect these cells normally. We had to use BF23 in these experiments because JK115 cannot adsorb T5. We found that JK115(ColIb) plated BF23 with an efficiency of about 1% compared with JK115 without the plasmid. Compared with the plating efficiency of 10^{-5}% for RM43, it appeared that the tolerance mutation had indeed made the cells less sensitive to the ColIb-induced inhibition of T5-like phage. It may be that the amount of intracellular colicin released by the phage simply overwhelms the tolerance mutation so that some inhibition occurs. Alternatively, the change in the cell membrane that makes JK115 tolerant also makes the membrane less sensitive to the phage-inhibiting protein. It is even possible that there is another, undetected mutation in JK115 that is increasing this cell's ability to grow phage in the presence of the Col-Ib plasmid.

We also used Tn*5*, a kanamycin resistance transposon (12), to obtain insertion mutants of the ColIb plasmid. Studies on the DNA from these mutants are now in progress, and until these are complete conclusions regarding the mutants must be viewed with caution. Pheno-

typic analysis shows, however, that all possible combinations of colicin production and phage inhibition exist. That is, we have isolated Tn5-containing ColIb plasmids that (i) produce colicin and inhibit phage (as does the wild-type plasmid), (ii) produce colicin and allow phage production (as does the previously isolated mutant plasmid in RM39 [5]), (iii) produce no colicin and allow phage production, and (iv) produce no colicin and inhibit phage production. Mutants of the third type would be predicted from the hypothesis of colicin-induced abortive infection. It may be, however, that these contain large deletions or have even lost the whole plasmid, transferring the kanamycin resistance to the chromosome. Another possibility is that the colicin gene and the phage inhibition gene are part of the same operon and that a regulatory gene controlling this operon has been affected in the third type of mutants. Isolation of the fourth type indicates that it is, indeed, possible to produce undetectable amounts of extracellular colicin and still inhibit phage production. It is, however, conceivable that these mutants are producing a fragment of the colicin molecule that can still act on the cell from within, but that cannot be excreted or cannot bind to other cells. A thorough analysis of the proteins produced by these strains will be necessary before any final conclusion can be reached. These studies, as well as a study of the DNA from these mutants, are currently in progress.

In summary, then, we have been unable to either prove or disprove the hypothesis of the colicin-caused abortive infection of T5. In each of three types of experiments, evidence supporting the hypothesis and evidence contradicting the hypothesis has been obtained. We may say, though, that if it is the colicin that is causing the abortive infection, this fact is surprisingly difficult to prove. Final proof, we feel, will come only from the cloning of the genes involved. Experiments to do this are underway.

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