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T-Even Bacteriophage-Tolerant Mutants of *Escherichia coli* B

I. Isolation and Preliminary Characterization

CHRISTOPHER K. MATHEWS

Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85721

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A general procedure is described for isolation of T-even phage-tolerant mutants of *Escherichia coli*. Two such mutants of *E. coli* B have been examined in some detail. These mutants adsorb T-even phages but are unable to release viable progeny. Under certain conditions, viability of the cells is completely unaffected by phage infection in one mutant, and there is but a slight decrease in colony-forming ability in the other. Phage deoxyribonucleic acid (DNA) is injected into these cells, as shown by the formation of phage-specific enzymes, but it is not degraded to acid-soluble material. Some phage DNA replication occurs in both strains. The mutants are both more resistant to ultraviolet light than is the parent strain.

To understand the role of host-cell functions in the replication of bacteriophages, it is useful to have phage-tolerant bacterial mutants, i.e., strains which can adsorb virus but are unable to propagate infectious phage. Such mutants have been described for ϕx174 (2, 3) and for λ (10) but not for the T-even phages. The latter phages contain so much genetic information of their own that one might not expect bacterial functions to play essential roles in phage replication. An additional factor which may have inhibited research in this area is the relatively high frequency with which *Escherichia coli* mutates to resistance to the T-even phages. Thus, most mutants which appeared "tolerant" by virtue of their ability to grow in the presence of phage would be so because of their inability to adsorb phage. I have circumvented this problem by the simple expedient of seeking strains which can grow in the simultaneous presence of two different T-even phages, T2 and T4. Since these phages adsorb to different sites on the *E. coli* cell wall (12), any bacterial strains resistant to adsorption by both phages would have to be double mutants. Thus, the background of resistant strains encountered in a mutant hunt is lowered by a large factor. This paper describes the isolation and some properties of two T-even phage-tolerant mutants of *E. coli* B.

MATERIALS AND METHODS

Bacteria were mutagenized by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by a modification of the method of Adelberg et al. (1). *E. coli* B, growing in nutrient broth, was harvested, washed in 0.1 M sodium citrate buffer (*p*H 5.0), and resuspended in the same buffer. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added at 100 μg/ml, and the cells were incubated at 37 C for 15 min. The cells were centrifuged and washed in sterile 0.9% NaCl, and a sample was diluted into nutrient broth and incubated overnight at 37 C.

For isolation of T-even-tolerant mutants, a sample of mutagenized bacteria containing between 10^7 and 10^8 viable cells was applied to a nutrient agar plate, by use of the pour-plate technique, in the presence of about 10^6 plaque-forming particles each of T2 and T4. The plates were incubated at 37 C. Between 20 and 50 bacterial colonies were seen on each plate against a background of confluent lysis. (It should be observed that no colonies were seen when a non-mutagenized stock was plated under identical conditions.) Ten colonies were picked and streaked on nutrient agar plates. These 10 stocks were checked for their ability to adsorb phages T2, T4, and T6 and for their failure to lose colony-forming ability after infection by one of these phages. Two strains were selected for further study and were named *E. coli* B *tet* 1 and *E. coli* B *tet* 2 (*tet* being the initials for T-even-tolerant).

Preparation of bromouracil-labeled phage and equilibrium density gradient centrifugation of CsCl were carried out as described by Murray and Mathews (9). Other methods, including ultraviolet (UV) irradiation, preparation of phages with labeled deoxyribonucleic acid (DNA), and assay of phage-induced enzymes, were carried out as described previously (8).

RESULTS AND DISCUSSION

T-even phage adsorption by tet mutants. The ability of B *tet* 1 and B *tet* 2 to adsorb T-even phages was checked in single infection of broth-

TABLE 1. *Adsorption of T-even phages by tet mutants*[a]

Bacteria	Per cent of unadsorbed phage		
	T2	T4	T6
Escherichia coli			
B	7.3	11.3	4.5
B *tet* 1	8.3	16.7	4
B *tet* 2	10	26.7	7.7

[a] Cells were grown at 37 C in nutrient broth to about 2×10^8 ml^{-1} and infected at an average multiplicity of 0.1. Unadsorbed phage titers were determined after chloroform treatment at 10 min after infection, with *E. coli* B as the plating bacteria.

grown cells at 37 C. As seen in Table 1, the mutants adsorbed T2 and T6 to about the same extent as *E. coli* B. T4 was not adsorbed quite as well, but more than 70% of the added phages were adsorbed in all cases studied. In other experiments, the mutants were found to adsorb T4 phage to a lesser degree than the parent strain when grown in minimal media. In one such experiment, *E. coli* B grown in M9 medium adsorbed 98% of added T4D within 5 min, whereas *tet* 1 and *tet* 2 adsorbed but 61 and 63%, respectively. Adsorption of T4 by the *tet* mutants is also quite poor in glycerol-Casamino Acids medium (5). Since satisfactory adsorption is seen in nutrient broth, this has been used for many of the preliminary experiments described in this report. However, for most of our biochemical experiments we are using glycerol-Casamino Acids medium in conjunction with infection by T6, because this system gives both satisfactory adsorption and a chemically defined environment for the infected cells.

Effects on cell viability. The two *tet* mutants differ somewhat from one another in response to infection by T-even phages. Infection of *tet* 1 by T4 in nutrient broth has no detectable effect on cell viability (Fig. 1), and infected cells continue to grow. On the other hand, infection of *tet* 2 by T4 leads to loss of colony-forming ability in one-third to one-half of the cells. However, in neither case are productive infections observed. As shown in Table 2, no infectious centers could be detected in cultures of either mutant infected with T4, nor was there any detectable formation of viable progeny phage.

Effects on viability in glycerol-Casamino Acids medium are somewhat variable, with survival after T6 infection of either strain ranging between 40 and 80% in different experiments.

Other properties. The *tet* mutants show no

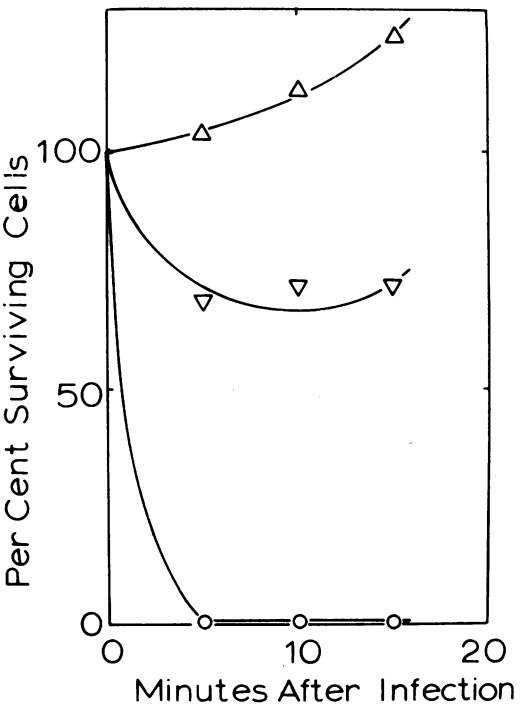

FIG. 1. *Survival of bacteria after infection with T4D. Cells were grown in nutrient broth to about* 3×10^8 ml^{-1} *and infected at an average multiplicity of 5. Surviving bacteria were assayed at the indicated times. Symbols:* ○, *E. coli B;* △, *B tet 1;* ▽, *B tet 2.*

absolute nutritional requirements, although growth in defined media is slower for both strains than for the parent strain. For example, *E. coli* B has a mass doubling time of 45 min in glycerol-Casamino Acids medium at 37 C, whereas the corresponding values for *tet* 1 and *tet* 2 are 78 and 69 min respectively. However, both mutants grow as rapidly in nutrient broth as does *E. coli* B. The mutant bacteria form smaller colonies on nutrient agar plates than does the parent strain, and microscopic examination shows cells of both *tet* 1 and *tet* 2 to be somewhat smaller than those of *E. coli* B. Unlike the analogous REP^- mutants affecting development of ϕx174 (2, 3) and λ (10), the *tet* mutants are considerably more resistant to irradiation by UV light than the parent strain, as shown in Fig. 2. Neither mutant is temperature-sensitive, as judged by relative ability to form colonies when incubated at 30 or 43 C.

One note of caution should be sounded in connection with work on T-even-tolerant strains. The mutants seem to revert with fairly high frequency, or else the few revertants formed can rapidly outgrow the mutants in a culture. At any rate, we find that stocks which have been transferred

TABLE 2. *Lack of productive infections in tet mutants infected with T4*[a]

Determination	Bacteria		
	B	B *tet* 1	B *tet* 2
Per cent of surviving bacteria	1.2	100	47.7
Per cent of cells yielding infective centers	100	<0.02	<0.02
Average burst size	220	<0.03	<0.05

[a] Cells were grown at 37 C to about 3×10^8 ml^{-1} in nutrient broth and infected with T4D at an average multiplicity of 5. At 5 min, the cells were centrifuged to remove most unadsorbed phage and resuspended in fresh broth. Five minutes after this, samples were removed for determination of surviving bacteria and infective centers (the latter corrected for the small amount of remaining unadsorbed phage). At 60 min after resuspension, samples were taken for determination of average burst size (based upon the cell concentration at time zero, and also corrected for unadsorbed phage). All platings were carried out with *E. coli* B.

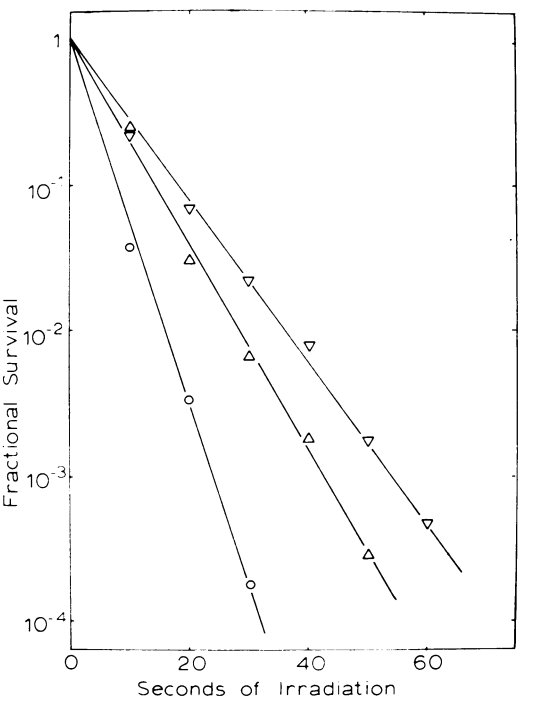

FIG. 2. *Ultraviolet light inactivation curves for E. coli B* (○); *B tet 1* (△); *and B tet 2* (▽).

several times tend to lose their tolerance to T-even phages. For this reason, we isolate stocks from single colonies at frequent intervals and check the phenotype before doing any biochemical experiments.

To date, we have not attempted any genetic analysis of either strain, particularly with respect to the question of whether single or multiple mutations are involved in generating the *tet* phenotype. The relatively high rate at which revertants appear suggests that single mutations are involved. Moreover, when a large colony-former was picked from a *tet* 2 culture as a possible revertant, it was found to have both the UV sensitivity and the susceptibility to T6 infection characteristic of *E. coli* B. Thus, at least in the case of *tet* 2, it would appear that a single mutation is responsible both for tolerance and for resistance to UV.

Can tolerance be overcome? One can easily isolate host-range mutants of T-even phages, i.e., strains which can productively infect bacteria which are resistant to adsorption by wild-type phage. One would like to know whether phage can undergo analogous mutations which would allow them to overcome the tolerance exhibited by the *tet* mutants. We have not yet investigated this question in detail. However, when as many as 10^6 viable particles of T2, T4, or T6 are plated on a lawn of either *tet* 1 or *tet* 2, no plaques are seen. Therefore, if T-even phages can mutate to overcome phage tolerance, the rate of spontaneous mutation is certainly quite low.

The same results were seen when phages T3 or T5 were plated on a lawn of either *tet* mutant; no plaques were seen even when 10^6 infectious phage were applied to a plate. Therefore, the two mutants may well be T-odd-tolerant as well as T-even-tolerant. However, since no definitive experiments have yet been carried out with the T-odd phages, we prefer to retain the name "T-even-tolerant" to define our mutants, at least for the present.

On the metabolic changes associated with T-even-tolerance. Why is infection abortive in cultures of the *tet* mutants? Clearly, an answer to this question will involve extensive studies of the reactions of phage development in tolerant cells. Such experiments are currently in progress and will be reported subsequently. To date, however, we have performed experiments which allow us to rule out three superficially plausible explanations of tolerance. First, one might expect that infections are abortive because *tet* mutants can degrade phage DNA. In this context, Smith et al. (11) showed that some strains of *E. coli* W survive T2 infection by virtue of an ability to break down phage DNA. However, we find no detectable degradation of labeled T4 DNA to acid-soluble

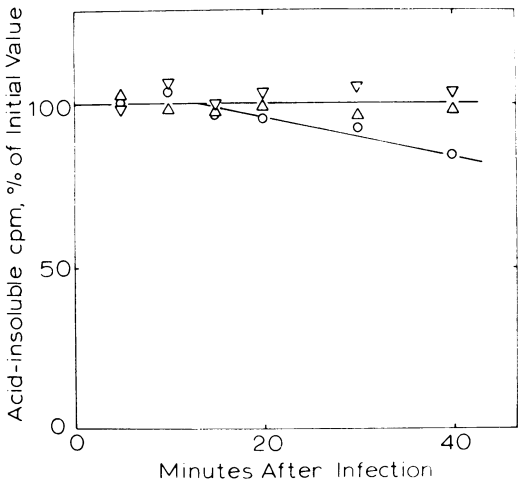

FIG. 3. *Degradation of parental phage DNA to acid-soluble material. Cells grown in nutrient broth to about 3 × 10³ ml⁻¹ were infected at an average multiplicity of 4 with ¹⁴C-uracil-labeled T4D. At the indicated times, 0.1-ml samples were removed and added to 1.0-ml portions of cold 5% trichloroacetic acid. The samples were passed through membrane filters (Millipore Corp.), which were than washed in cold 5% trichloroacetic acid, dried, and counted in a liquid scintillation counter. Symbols:* ○, *E. coli B;* △, *B tet 1;* ▽, *B tet 2.*

material (Fig. 3) even though some such breakdown was seen in infected cultures of *E. coli* B.

Another possible explanation of tolerance is that the bacterial cell surface might be altered in such a way that phage DNA is not injected into the cell. Although DNA injection has not been tested directly, we do find that phage-induced enzymes are synthesized by both *tet* mutants in T6-infected cells, a phenomenon which could occur only if DNA were injected. Synthesis of the early enzymes, dCMP hydroxymethylase and dihydrofolate reductase, occurs to the extent of about 40% of control values although the reductase activity decreases to preinfection levels late in infection (Fig. 4). A small amount of the "late" enzyme, lysozyme, was also synthesized. Although at first glance it may seem surprising that a late phage function seems to be expressed in abortively infected cells, it must be stated that there is no assurance that lysozyme is synthesized in all of the infected cells. Cell survival was only 39% in the *tet* 1 culture used for this experiment (Table 3) and 80% with *tet* 2. It is conceivable that lysozyme is synthesized only in those cells which do not survive. In any case, we should observe that, just as seen with T4, infection of all cells by T6 is abortive, as judged both by the failure of any cells

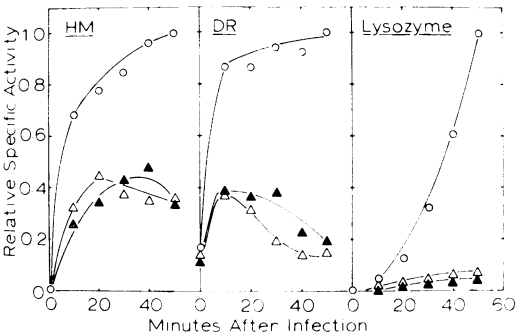

FIG. 4. *Phage-induced enzyme synthesis. Cells were grown in glycerol-Casamino Acids medium to about 3 × 10³ ml⁻¹ and infected with T6 at an average multiplicity of 5. Samples were removed at the indicated times and assayed for dCMP hydroxymethylase (HM), dihydrofolate reductase (DR), and lysozyme. Symbols:* ○, *E. coli B;* △, *B tet 1;* ▲, *B tet 2.*

TABLE 3. *Lack of productive infections in tet mutants infected with T6*[a]

Determination	Bacteria		
	B	B *tet* 1	B *tet* 2
Per cent of phage not adsorbed	3.3	6.5	4.2
Per cent of surviving bacteria	4.6	38.7	79.6
Per cent of cells yielding infective centers	100	<0.06	<0.06
Average burst size	274	<0.30	<0.10

[a] Cells were grown in glycerol-Casamino Acids medium to about 3 × 10⁸ ml⁻¹ and infected with T6 at an average multiplicity of 5 (these were the same cultures used in the experiment described in Fig. 4). Samples were taken at 10 min after infection for assay of unadsorbed phage, infective centers, and surviving bacteria, and at 60 min for determination of average burst size, as described in the legend to Table 2.

to form infective centers or of viable phage progeny to be formed.

As a third explanation for tolerance, one might propose that the *tet* mutants are unable to support phage DNA replication. Suppose, for example, that the structure of the bacterial membrane is altered such that parental phage DNA cannot attach to it as a prelude to replication. One cannot test DNA replication in this system by following incorporation of isotopic DNA precursors, for we have not yet established the extent to which bacterial functions are depressed in *tet* mutants soon after infection. Certainly the shutoff of bacterial functions, even if it occurs, is not permanent;

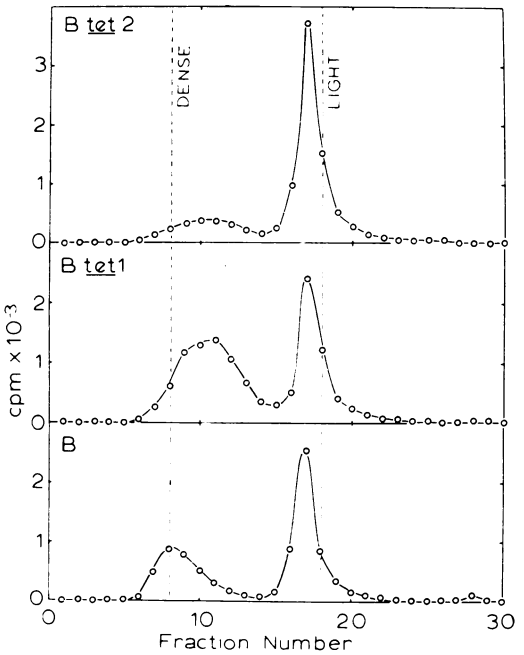

FIG. 5. *DNA replication. Cultures initially at cell concentrations of 3 × 10⁷ ml⁻¹ to 5 × 10⁷ ml⁻¹ in glycerol-Casamino Acids medium were grown in 37 C for about one generation. BUDR was then added to 20 μg/ml, along with 30 μg of 5-fluorodeoxyuridine per ml, the latter added to block endogenous thymidylate synthesis and promote BUDR incorporation. Uracil was also added at 20 μg/ml. The cells were density-labeled by growth for a further two generations, whereupon 5-ml cultures were infected at a multiplicity of 8 with T6 containing ¹⁴C-uracil-labeled DNA. Twenty minutes after infection, the cultures were centrifuged to remove unadsorbed phage and resuspended in 2 ml each of tris(hydroxymethyl)aminomethane (Tris)-glucose salts (9). Lysis was carried out by the low-temperature procedure of Frankel (4). Tritium-labeled T4 phage preparations were added as density markers to each culture: a sample of "light" phage prepared by labeling with ³H-thymidine, and a sample of "dense" phage prepared by labeling with ³H-BUDR. The lysates were then extracted with two successive 4-ml portions of phenol saturated with Tris-glucose salts. The extraction was carried out by gentle rolling in a cold room, and precautions were taken to minimize hydrodynamic shear. The DNA samples were dialyzed exhaustively against standard saline citrate, and samples were prepared for CsCl gradient centrifugation (9). The gradients were centrifuged at 37,000 rev/min for 52 hr. The vertical dashed lines on the graph indicate the positions of the dense and light ³H-labeled marker DNA values, as indicated. It was impossible to monitor cell survival in this experiment, since bacterial viability was severely affected by growth in BUDR. However, infection of the tet mutants was abortive, as shown by the absence of infective centers and the failure of detectable progeny phage to be formed.*

otherwise, the cells could not survive to form colonies. Accordingly, I tested for phage DNA synthesis by examining the extent to which ¹⁴C-labeled parental T6 DNA increased in density after infection of cells grown for two generations in the presence of 5-bromodeoxyuridine (BUDR). As discussed by Kozinski and Felgenhauer (7), this approach provides a more sensitive test of DNA replication than does isotope incorporation, even in the absence of complications caused by possible incomplete shutoff of host-cell functions.

Significant DNA replication occurs in the *tet* mutants (Fig. 5). By 20 min after infection, the time at which cells were harvested in this experiment, the proportion of parental DNA which had undergone replication is actually greater in B *tet* 1 than in B, although considerably less in B *tet* 2. Similar results were seen in other experiments (not shown) involving collection of the infected cells at 15 rather than 20 min.

Examination of Fig. 5 shows that the replicated parental DNA in infection of *E. coli* B assumes a density comparable to that of heavy phage DNA (i.e., fully labeled with BUDR). This is, of course, expected from the fragmentation and dispersion of parental DNA among progeny DNA molecules, as described by Kozinski (6). On the other hand, replicated parental DNA in infection by either *tet* mutant assumes a hybrid density. Since the DNA samples used in this experiment were isolated by a low-shear procedure minimizing DNA degradation, one can explain this result either by postulating that dispersion of parental label does not occur, or else that dispersion does occur but intracellular phage DNA undergoes extensive breakage in the *tet* mutants. Resolution of this question must await the results of studies on the sedimentation rates of intracellular phage DNA in infection of the *tet* mutants. One should note also that the experiment of Fig. 5 says nothing about the relative extents of DNA replication in the various cultures, but only shows that some DNA replication occurs. It is conceivable that only a single round of DNA replication occurs in the *tet* mutants.

I have thus described a method for isolation of bacterial mutants which can adsorb T-even phages but cannot support virus growth. Several early phage functions are expressed, including early enzyme synthesis and some DNA replication. The metabolic step at which phage multiplication stops is unknown.

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