

Characterization of *Escherichia coli* Mutants Tolerant to Bacteriocin JF246: Two New Classes of Tolerant Mutants

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Several hundred independent bacteriocin-tolerant mutants have been isolated without mutagenesis from three strains of *Escherichia coli*. On the basis of patterns of sensitivity to eight different colicins, over 85% of these mutants could be grouped into four classes. Two classes of mutants, class A and class B, are equivalent to *tolA* and *tolB* type mutants. We found *tolA* and *tolB* mutants were sensitive to the antibiotic bacitracin. The other two classes of bacteriocin-tolerant mutants, class F and class G, are distinguished from other types of colicin-tolerant mutants on the basis of sensitivity to colicins, dyes, detergents, antibiotics, and chelating agents. The mutation in class F and class G mutants is located between 21 to 23 min on the *E. coli* chromosome. We propose to designate the loci of these mutations as *tolF* and *tolG*, respectively.

Colicin-tolerant mutants in *Escherichia coli* have been isolated in a number of laboratories, usually by treating a population of sensitive cells with a particular colicin and looking among the survivors for those which adsorb the colicin but are not killed by it (2, 3, 10, 14-16). Often these mutants have become tolerant to other colicins in addition to the colicin used as the selection agent. Multiple colicin tolerance does not follow patterns predicted on the basis of common mode of action or receptor. For example, a single mutation *tolB* results in tolerance to colicins E2, E3, and K, where each of these colicins has a different mode of action and colicins E2 and E3 adsorb to a receptor distinct from the colicin K receptor (14). These observations are consistent with the current hypothesis that the mechanism of action of colicins involves an interaction of the colicin with the cell membrane and mutations affecting the cell membrane result in tolerance to single or multiple colicins.

The evidence that colicin-tolerant cells carry altered cell membranes is primarily indirect. For example, some tolerant mutants show an increased sensitivity to one or more of a variety of agents such as dyes, detergents, and antibiotics (2, 3, 14, 15), a fact which is often taken as a reflection of increased permeability or decreased stability of the cell membrane. In addition, examination of the proteins in the mem-

brane fraction from certain tolerant mutants indicates that these factors may lack proteins found in similar preparations from the wild type (17).

Some of the mutations leading to tolerance have been mapped. Two loci are near the *gal* locus (*tolA*, *tolB*) (1, 14, 15), a third is near the *metC* locus (*tolC*) (21), and two others, *tolD* and *tolE*, are close to 20 min on the *E. coli* chromosome (2, 5). *tolA* mutants are tolerant to all colicins tested (A, E1, E2, E3, and K), whereas *tolB* mutants are sensitive to colicin E1 but tolerant to the other colicins tested (A, E2, E3, K) (14, 16). *tolC* mutants are tolerant to colicin E1 alone (14, 21) and *tolD* and *tolE* mutants are tolerant to colicins E2 and E3 (2). In addition, mutation in the *cet* locus near *serB* confers tolerance to colicin E2 (19).

Bacteriocin JF246 produced by *Serratia marcescens* strain JF246 kills certain strains of *E. coli* (7). It has a mode of action similar to those described for colicins A, E1, or K and inhibits all macromolecular synthesis in sensitive cells (6).

This investigation describes the isolation and characterization of several hundred spontaneous, independent bacteriocin-tolerant mutants from each of three *E. coli* strains. These studies were undertaken with the expectation that new classes of *E. coli* bacteriocin-tolerant mutants would be isolated by using bacteriocin JF246 as

the selection agent and that these mutants might be useful in the study of colicin action and membrane structure. The majority of our tolerant mutants fell into four classes on the basis of their patterns of sensitivity to eight different colicins. Two of these classes were identical with the *tolA* and *tolB* mutants described by others. The remaining two major classes (class F and class G) exhibit distinct patterns of sensitivity to colicins, detergents, and antibiotics. The mutations in these strains are in an area of the chromosome close to the *tolD* and *tolE* loci. We propose to denote the loci of these mutations as *tolF* and *tolG*.

MATERIALS AND METHODS

Organisms and culture media. The bacterial strains used in this investigation are described in Table 1. Normally, the bacteria were grown in rich medium (PPBE) which contained 10 g of proteose peptone no. 3 (Difco), 2 g of beef extract (Difco), and 5 g of NaCl per liter. PPBE agar contained 1.5% agar and PPBE soft agar contained 0.75% agar. For growth of phage P1, L broth, and L agar (11) containing 2.5 mM CaCl₂ were used. The minimal medium used was that described by Vogel and Bonner (20) supplemented with glucose or galactose at 0.2%, streptomycin (100 µg/ml), and the appropriate growth requirements.

Preparation and assay of bacteriocins. All bac-

teriocins except bacteriocin JF246 were crude preparations from mitomycin C-induced broth cultures of the appropriate colicinogenic strain. Cells were first grown in PPBE medium to a density of approximately 5×10^8 cells per ml and mitomycin C (Sigma) was added to a final concentration of 2 µg/ml. After an additional 4 to 8 h of incubation at 37 °C, the cells were collected by centrifugation and the cell-bound colicin was extracted with 1.0 M NaCl in 0.02 M potassium phosphate buffer, pH 7.0. Solid (NH₄)₂SO₄ was then added to the NaCl extract to 75% saturation and the precipitate was collected by centrifugation and suspended in 0.02 M potassium phosphate buffer, pH 7.0. This procedure yielded crude colicin preparations which had a milky appearance and were stable when frozen. Purified bacteriocin JF246 was prepared as previously described (7).

All bacteriocins were titered by the spot test method previously described where one unit of bacteriocin activity was defined as the reciprocal of the highest dilution that completely inhibited the growth of the indicator strain JF135 (6). Typically, the activity (units per milliliter) of the various bacteriocin preparations was as follows: colicin A, 640,000; colicin C, 64,000; colicin E1, 800; colicin E2, 128,000; colicin E3, 160,000; colicin Ia, 160; colicin Ib, 160; colicin K, 64,000; bacteriocin JF246, 320,000.

Isolation of bacteriocin-tolerant mutants. Independent mutants tolerant to bacteriocin JF246 were isolated without mutagenesis from *E. coli* K12 strains HfrH (JF404), W3110 (JF363), and CSH75. Cells were first grown in PPBE medium in tubes (13 by 100 mm)

TABLE 1. Bacterial strains^a

Strain	Genotype and comments	Source or reference
<i>E. coli</i> K12 derivatives		
JF135	W3110, F ⁻ , <i>leu</i> , <i>trp</i> , <i>ilv</i> , λ ⁻ (colicin sensitive indicator)	6
JF363	W3110, F ⁻ , <i>proC</i> , <i>trp</i> , <i>his</i> , <i>thyA</i> , <i>metB</i> , <i>strA</i> , λ ⁻	L. Soll
JF396	W3110, F ⁻ , <i>leu</i> , <i>trp</i> , <i>ilv</i> , <i>strA</i> , λ ⁻	JF135
JF404	Hfr (Hayes), <i>thyA</i> , λ ⁻	B. Low
JF418	Hfr strain KL16, <i>ser</i> , <i>thi</i> , <i>recA</i> , λ ⁻	B. Low
JF430	<i>gal</i> , <i>pro</i> , <i>met</i> , <i>recA</i> , F ⁺ <i>gal</i> ⁺ , <i>tolA</i> ⁺ B ⁺	A. Bernstein strain BN37, 1
JF431	<i>thr</i> , <i>leu</i> , <i>bio</i> , <i>gal</i> , <i>tolA617</i> , F ⁺ <i>gal</i> ⁺ , <i>tolA617</i>	A. Bernstein strain BN45, 1
JF432	<i>thr</i> , <i>leu</i> , <i>bio</i> , <i>gal</i> , <i>tolB515</i> , F ⁺ <i>gal</i> ⁺ , <i>tolB515</i>	A. Bernstein strain BN46, 1
JF435	<i>ara</i> , <i>proC</i> , <i>lacY</i> , <i>purE</i> , <i>gal</i> , <i>trp</i> , <i>recA</i> , <i>argG</i> , <i>malA</i> , <i>mtl</i> , <i>xyl</i> , <i>ilv</i> , <i>met</i> , <i>thi</i> , <i>strA</i> , λ ⁻	CSH75 ^b
CSH75	<i>ara</i> , <i>leu</i> , <i>proC</i> , <i>lacY</i> , <i>purE</i> , <i>gal</i> , <i>trp</i> , <i>his</i> , <i>argG</i> , <i>malA</i> , <i>mtl</i> , <i>xyl</i> , <i>ilv</i> , <i>met</i> , <i>thi</i> , <i>strA</i> , λ ⁻	Cold Spring Harbor
Bacteriocin producers		
JF246	<i>S. marcescens</i> , <i>trp</i> , (produces bacteriocin JF 246)	6
JF367	<i>E. coli</i> Y20, <i>thr</i> , <i>leu</i> , <i>thi</i> (Col E1)	D. Feingold
JF371	<i>E. coli</i> CR34 <i>tonA21</i> (Col E2)	D. Helinski
JF322	<i>E. coli</i> CR34 <i>tonA21</i> (Col E3)	D. Helinski
JF384	<i>E. coli</i> K235 (Col K)	S. Luria
JF385	<i>E. coli</i> 06: H16, 23 (Col A)	S. Luria
JF390	<i>Paracoli</i> Ca57 (Col C)	J. Papavassilou
JF538	<i>E. coli</i> K12 W3110λ ⁻ (Col Ia)	J. Konisky strain JK16
JF539	<i>E. coli</i> K12 W3110λ ⁻ (Col Ib)	J. Konisky strain JK20

^a Genetic symbols are described by Taylor and Trotter (18).

^b A *recA his*⁺ recombinant was selected following conjugation of strain JF418 with strain CSH75. Next, a *leu*⁺ transductant was selected using phage P1 grown on strain JF363.

from a small inoculum (10^3 cells/ml) to a concentration of approximately 10^8 cells per ml. Each tube was then treated for 20 min with bacteriocin JF246 at a final concentration of 500 to 1,000 U per ml. The surviving bacteria were plated at a dilution of 10^{-1} on PPBE agar. After an overnight incubation at 37 C several colonies were picked from each plate and tested to distinguish tolerant and resistant mutants (see below). Finally, a single tolerant mutant was selected to represent each independent culture.

Plate assay for the presence of bacteriocin receptor. Bacteriocin-tolerant mutants are distinguished from resistant mutants by the presence of a specific bacteriocin receptor on the surface of tolerant cells. The presence of a receptor for a bacteriocin was demonstrated by first placing 2 to 5 μ liters of a dilution containing approximately 10^4 cells per ml of the culture to be tested on the surface of a PPBE plate. As many as 16 mutants could be tested on a single plate. After allowing the cells to develop into a macrocolony by incubation for 12 to 18 h at 37 C, the cells were killed by CHCl_3 vapor and the plate was overlaid with 3.0 ml of PPBE soft agar containing 500 U of a bacteriocin and streptomycin (100 $\mu\text{g/ml}$) to inhibit the growth of any cells not killed by the CHCl_3 vapor. After a 6-h incubation at 37 C to allow adsorption of bacteriocin by the receptors on the killed cells in the macrocolony, the plate was overlaid with 3.0 ml of PPBE soft agar containing approximately 10^7 cells of the indicator strain JF396. After a final incubation of 12 to 18 h, the plates were scored. The macrocolonies formed by bacteriocin-tolerant mutants retained the specific bacteriocin receptor and were capped by a circle of growth of the indicator strain, whereas no growth of the indicator was seen over the macrocolonies formed from bacteriocin-resistant mutants that lack the receptor.

Although this procedure would score as tolerant any mutant that excreted a bacteriocin inactivator, we saw none of the type described by Guterman and Luria (8). Nor did we find any bacteriocin inactivator in any of 40 culture supernatant fluids tested from different tolerant mutants.

Patterns of colicin sensitivity of mutants tolerant to bacteriocin JF246. The sensitivity of mutants selected as tolerant to bacteriocin JF246 to eight colicins was determined by placing 2 to 5 μ liters of each dilution of the colicin on the surface of a PPBE plate overlaid with 3.0 ml of PPBE soft agar containing approximately 10^7 cells of the mutant to be tested. Several different colicins were tested on a single plate. After overnight incubation the colicin sensitivity of the mutants was determined by noting which colicin completely inhibited growth of the mutant.

Sensitivity to other agents. The sensitivity of the tolerant mutants to sodium deoxycholate (DOC), sodium dodecyl sulfate (SDS), disodium ethylene diaminetetraacetic acid (EDTA), rifamycin, eosin yellow, methylene blue, and acridine orange was measured by applying 2 to 5 μ liters of a dilution containing about 10^4 cells per ml of the mutant to be tested on a plate containing PPBE agar supplemented with, for example, 1% sodium deoxycholate. After overnight incubation at 37 C, the growth of the mutant on the

plate containing sodium deoxycholate was compared with the growth of the mutant on a similar plate containing no additions. By using this procedure the sensitivity of as many as 50 mutants to a given agent could be tested on a single plate.

Alternatively, sensitivity was determined by diluting an overnight broth culture 10^{-3} into PPBE broth containing one of the above agents. After 6 to 8 h of incubation at 37 C with aeration, the cultures were scored for growth by comparison of the absorbance at 650 nm with a control culture containing no additions.

The sensitivity to the antibiotics novobiocin and bacitracin was determined by using the commercially available Sensi-Discs obtained from BBL, Cockeysville, Md. A single disk was placed on a PPBE soft agar overlay containing approximately 10^7 cells. After overnight incubation, sensitivity was scored by noting the presence of a distinct zone of inhibition (diameter approximately 10 mm) surrounding the Sensi-Disc. Resistance was expressed by no detectable inhibition by the antibiotic.

The sensitivity of the tolerant mutants to ultraviolet (UV) light was determined by spreading approximately 100 cells on each of two PPBE plates. One of the plates was exposed to a dose (approximately 800 ergs/ mm^2) of UV irradiation sufficient to reduce the viability of the control culture approximately 20% (80% survival). After overnight incubation at 37 C, the number of colonies on the two plates was compared. Mutants were scored as sensitive where the number of colonies on UV-irradiated plates was less than 25% that found on the control plates (25% survival).

Genetic procedures: testing for mating ability. Before each mating experiment a good donor colony was isolated. This was necessary because the Hfr property was not well expressed in certain tolerant mutants. To select a good donor, streaks were first made from each tolerant mutant and 50 to 100 well-isolated single colonies were picked with sterile toothpicks and tested for mating ability by making a stab inoculation onto PPBE agar plates in a grid. After a 6-h incubation, the grids were replicated onto supplemented minimal plates containing streptomycin and all the supplements required for growth of strain CSH75 except leucine. These plates had been spread with approximately 5×10^7 cells of strain CSH75. After 24 to 36 h of incubation at 37 C, the colonies which could donate *leu*⁺ to strain CSH 75 could be identified by the growth of *leu*⁺ recombinants on the minimal plate.

Mapping by interrupted mating. Hfr \times F⁻ mating experiments were carried out at 37 C in PPBE broth supplemented with 0.2% glucose by mixing about 2×10^7 Hfr cells per ml with 2×10^8 F⁻ cells per ml. After dilution of samples of the mating mixture 10- to 100-fold, mating was interrupted by vigorous shaking by using the device described by Low (12). The cultures were then plated on minimal medium containing the appropriate supplements.

Mapping by gradient of transmission. Mapping by the gradient of transmission method (4) used the same mixture of donor and recipient cells described above for interrupted mating. After an incubation at

37 C for 5 min without shaking, the mating mixture was gently diluted 100-fold into prewarmed medium and the mating continued for 2 h without shaking. At this time the mating was interrupted and streptomycin was added to a final concentration of 100 µg/ml to inhibit the growth of the donor strain. The culture was then incubated at 37 C for an additional 75 to 90 min (about two generations) and finally plated on selective medium.

Preparation of *recA* derivatives of tolerant strains. The *recA* mutation was introduced into bacteriocin-tolerant mutants derived from strain CSH75 by first mating with an Hfr *recA* donor, JF418. The mating mixture was interrupted after 50 min and plated on selective medium lacking histidine. After 48 h of incubation at 37 C both large and small *his*⁺ recombinant colonies were seen. Approximately 20 to 30 small *his*⁺ recombinant colonies were picked, purified, and a single colony of each recombinant was picked and grown up on a PPBE agar plate. This plate was next replicated to two PPBE agar plates, one of which was irradiated with UV light (total dose, 800 ergs/mm²). The *his*⁺ *recA* recombinants did not grow on the irradiated plate. The *his*⁺ *recA* bacteriocin-tolerant recombinants were then picked from the unirradiated plate and tested to show that other markers such as *trp*⁺ and *gal*⁺ has not been incorporated. The pattern of sensitivity of the *his*⁺ *recA* bacteriocin-tolerant recombinants to colicins and detergents was also tested to confirm that their response was identical to that of the parental strain.

Complementation studies. F'*gal*⁺ partial diploids containing the *tolAB* loci on the episome were prepared starting with *recA* tolerant mutants derived from strain CSH75 by first mixing 10⁷ F'*gal*⁺ donor cells with 10⁸ recipient cells. After a 15-min incubation period at 37 C, the culture was diluted in saline and plated on selective medium containing galactose and streptomycin but lacking threonine. The *gal*⁺ colonies were purified and characterized by their nutritional requirements, their sensitivity to UV irradiation and phage f2, and by their ability to donate F'*gal*⁺ into the *recA* recipient strain JF439.

Transduction. Transduction with phage P1(kc) was done by using the procedure described by Lennox (11). Phage, prepared by the plate lysis method, were mixed with recipient cells in L broth, and, after allowing 20 min for phage adsorption, the cells were washed with Vogel-Bonner medium (20) and spread on selective plates.

RESULTS

Patterns of colicin sensitivity. Starting with each of three parental strains, a large number of independent mutants tolerant to bacteriocin JF246 have been isolated. When the sensitivity of each of these mutants to eight colicins was tested, the majority fell into four classes. The patterns of colicin sensitivity of these four classes of mutants are summarized in Table 2. Tolerant mutants that displayed other patterns of colicin sensitivity have not yet been further

characterized, but many of these were similar to the partially tolerant phenotype previously described and labeled *tolP* by Bernstein, Rolf, and Onodera (1).

The total number of mutants isolated from each parent as well as the relative number of mutants in each of the four major classes is shown in Table 3. These four classes accounted for 80 to 92% of the tolerant mutants isolated from each parent. Class A and class B mutants were the most frequently isolated. They exhibited patterns of colicin sensitivity similar to the *tolA* and *tolB* type mutants described by a number of authors (9, 14, 16).

The remaining two major classes of mutants isolated as tolerant to bacteriocin JF246, labeled class F and G in Table 2, has distinct patterns of colicin sensitivity. Class G mutants were tolerant only to bacteriocin JF246. Class F mutants were tolerant or partially tolerant to colicins A, K, and bacteriocin JF246, and sensitive to colicins C, E1, E2, E3, Ia, and Ib.

Sensitivity to antibiotics, detergents, and dyes. Tolerant mutants often have an increased sensitivity to a variety of agents including detergents, dyes, antibiotics, and chelating agents. For example, the *tolA* and *tolB* type mutants are markedly more sensitive to DOC and EDTA than the wild type (14, 16). The sensitivity of the four classes' tolerant mutants to a variety of agents was tested. For these tests several mutants representing each class from each parental strain were chosen. The results, summarized in Table 4, showed that each of the four classes of mutants had a distinct pattern of response to the agents tested. The results were generally consistent even where mutants of a given class were derived from different parental strains, although mutants derived from strain CSH75 were more sensitive to SDS than those isolated from strains JF404 or JF363. All class A and class B mutants tested were sensitive to bacteriocin; most (23/25) class F and G mutants were sensitive to novobiocin.

Mapping the mutation in class A and class B strains. The mutants of class A and class B appeared to be similar to strains that carried a mutation in the *tolA* and *tolB* loci first described by Nagel de Zwaig and Luria who showed that the *tolA* and *tolB* loci were near the *gal* locus (14). Transduction tests using phage P1 showed that the mutation in four independent isolates of our class A and B strains was linked to the *gal* locus (Table 5).

Complementation studies. To confirm that our class A and class B mutants were identical with *tolA* and *tolB* mutants isolated as tolerant to colicins A, E1, E3, and/or K, we prepared

partial diploids using *F'*gal episomes carrying either *tol*⁺, *tolA*, or *tolB* mutations. For these studies, *recA* derivatives of 2 to 5 separate mutants representing each of the four classes were prepared. This procedure served two purposes. First, the *recA* mutation stabilized the *F'* by preventing its integration into the chromosome so that *gal*⁻ *recA* *F'*gal⁺ partial diploids could be maintained by growth of the strains media containing galactose as the carbon source. Second, the *recA* mutation prevented recombination between the *tol* locus on the episome and the *tol* locus on the chromosome making the results of complementation studies easier to interpret.

A study of the patterns of colicin sensitivity of representative *F'*gal⁺*tol* partial diploids, summarized in Table 6, showed that the class A mutants were complemented by either *F'*gal⁺*tol*⁺ or *F'*gal⁺*tolB* episomes, but not by the *F'*gal⁺*tolA* episome. The class B mutants similarly were complemented by the *F'*gal⁺*tol*⁺ or *F'*gal⁺*tolB* episome. These results confirm

that our class A and class B mutants are equivalent to the *tolA* and *tolB* mutants isolated by others. The *F'*gal⁺*tol*⁺ episome was unable to complement class F or class G-tolerant mutants (Table 6).

Mapping the mutation in class F and class G strains. P1 phage transduction studies showed that neither the tolerant mutation in the class F nor class G mutants were linked (<0.5%) to the *gal* locus. These mutations were mapped first by interrupted mating and then by the gradient of transmission method.

For the interrupted mating studies, individual tolerant mutants derived from strain Hfr H were mated with an appropriately marked strain and the mating mixture was interrupted

TABLE 2. Colicin sensitivity of mutants tolerant to bacteriocin JF246

Parental strain or representative mutant	Class	Colicin ^a								
		A	bc	C	E1	E2	E3	K	Ib	
JF404	Wild type	S	S	S	S	S	S	S	S	S
JF404-3a	A	T	T	T	T	T	T	T	T	S
JF404-8a	B	T	T	S	S	T	T	T	T	S
JF404-2a	G	S	T	S	S	S	S	S	S	S
JF404-4a	F	T	T	S	S	S	S	T	S	S

^a Concentration of colicin used (units/milliliter): colicin A, 1,000; bc JF246, 1,000; colicin C, 1,000; colicin E1, 100; colicin E2, 800; colicin E3, 1,000; colicin K, 800; colicin Ib, 80. Symbols: S, sensitive; T, tolerant.

TABLE 3. Distribution of tolerant mutants in various classes

Parental strain	Class	No. of independent isolates	Percentage ^a
JF404	A	183	32
	B	218	39
	G	78	14
	F	39	7
	Unclassified	48	8
JF363	A	106	32
	B	135	41
	G	23	7
	F	9	3
	Unclassified	56	17
CSH75	A	27	42
	B	8	12
	G	11	17
	F	12	18
	Unclassified	7	11

^a Percentage of total tolerant mutants isolated from a given parent.

TABLE 4. Sensitivity of tolerant mutants to various treatments

Class ^a	DOC (1%)	SDS (1%)	EDTA (2 × 10 ⁻³ M)	Rifamycin (4 µg/ml)	Novobiocin (30 µg)	Bacitracin (10 U)	Eosin yellow (400 µg/ml)	Acridine orange (100 µg/ml)	Methylene blue (100 µg/ml)	Ultra-violet irradiation (80 ergs/mm ²)
Wild type	R ^b	R	R	R	R	R	R	R	R	R ^c
A	S	R	S	R/S ^d	R	S	R	R	R	R
B	S	R	S	R/S	R	S	R/S	R	R	R
G	R	R	S	R/S	S	R	S	R	R	R
F	S	R	R	R/S	R/S	R	R	R	R	R

^a At least three tolerant mutants of each class derived from each parental strain were tested.

^b Symbols: R, resistance; S, sensitivity; R/S, individual strains gave varied response.

^c Viability of the parental strains following UV irradiation was 72 to 88% of the unirradiated control. The viability of the tolerant strains varied from 67 to 90%.

^d Sensitivity of tolerant mutants to rifamycin depended upon the parental strain. All mutants isolated from CSH75 were sensitive whereas mutants from JF363 or JF404 were not.

every 120 s. The appearance of recombinants for several selected markers was measured after various times of mating and the map position of the tolerance locus was estimated by determination of the fraction of tolerant recombinants for each of the selected markers. The results showed that the mutations in class F and class G were between *gal* and *trp* on the *E. coli* chromosome.

To determine the location of the mutation in class F and class G strains more precisely, *purE*⁺ or *proC*⁺ recombinants were selected after mating selected mutants with strain CSH75. The gradient method of mating was used which allows time for zygote formation and recombination before plating on selective media.

For these experiments we used a multiply marked F⁻ strain as the recipient and the tolerant derivatives of strain HfrH as donors, a relatively early marker, such as *proC* or *purE* was used as the selected marker and from 476-750 Pro⁺Str^r or Pur⁺Str^r recombinants picked and tested for co-transfer of other mark-

ers including tolerance. The data from three crosses involving class F mutants and two crosses involving class G mutants are summarized in Table 7. When these data were plotted on a semilog scale, where the log of the relative frequency of co-transfer of an unselected marker was plotted against the map position in minutes, a straight line resulted (Fig. 1). When the relative frequency of co-transfer of tolerance was plotted on this line we see that for each of the crosses depicted, the map position of the mutation in the class F or class G strains was at approximately 21 to 23 min on the standard *E. coli* map (18).

DISCUSSION

A bacteriocin first interacts with a sensitive cell by attaching to a specific receptor on the cell surface. A mutation to bacteriocin resistance alters this receptor and prevents adsorption of bacteriocin. In bacteriocin-tolerant mutants the receptor is apparently unchanged, for these mutants adsorb bacteriocin as well as the wild type. However, the mutation in these strains results in a reduced probability of a killing effect of a bacteriocin.

A mutant selected as tolerant to a given bacteriocin often simultaneously becomes tolerant to a number of different, unrelated bacteriocins. The patterns of tolerance in these mutants do not follow patterns predicted on the basis of a common receptor or a common mode of action. This result has led to the postulate that the mutations leading to tolerance effect the organization of the components of the cell membrane (15). Since the membrane is involved in a number of cellular functions, there may be a number of potential mutations which lead to an alteration in the membrane and a

TABLE 5. Co-transduction by phage P1 of bacteriocin tolerance with the *gal* locus in *E. coli*^a

Class	Parent	Representative mutants studied	Range of co-transduction frequency (%)
A	JF404	3a, 13c, 15a, 18a	32-58
B	JF404	1a, 8a, 10a, 13a	60-65

^a Strain CSH75 was used as a recipient. In each transduction, from 74 to 100 *gal*⁺ transductants were picked and cross streaked against bacteriocin JF246 to determine co-transduction of the tolerance locus.

TABLE 6. Complementation studies using F' *gal* partial diploids prepared from *recA* derivatives of each class of tolerant mutant

Recipient strain	Phenotypic class of recipient ^a	Donor strain	Phenotypic class of donor	Genotype of partial diploid ^b (relevant markers)	Phenotypic class of partial diploid
CSH75- <i>tol</i> 18	A	JF430	Wild type	<i>gal</i> ⁻ , Class A, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolA</i> ⁺ B ⁺	Wild type
		JF431	A	<i>gal</i> ⁻ , Class A, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolA</i>	A
		JF432	B	<i>gal</i> ⁻ , Class A, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolB</i>	Wild type
CSH75- <i>tol</i> 13	B	JF430	Wild type	<i>gal</i> ⁻ , Class B, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolA</i> ⁺ B ⁺	Wild type
		JF431	A	<i>gal</i> ⁻ , Class B, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolA</i>	Wild type
		JF432	B	<i>gal</i> ⁻ , Class B, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolB</i>	B
CSH75- <i>tol</i> 5	G	JF430	Wild type	<i>gal</i> ⁻ , Class G, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolA</i> ⁺ B ⁺	G
CSH75- <i>tol</i> 20	F	JF430	Wild type	<i>gal</i> ⁻ , Class F, <i>recA</i> F' <i>gal</i> ⁺ , <i>tolA</i> ⁺ B ⁺	F

^a Phenotypic class was determined by pattern of sensitivity to eight different colicins.

^b Partial diploids were tested for their sensitivity to UV irradiation sensitivity to male-specific phage f2 and their ability to donate F'*gal*⁺ into a *recA* recipient, JF439.

TABLE 7. Mapping class E and F tolerant mutations by gradient method

Cross	Donor ^a	Tolerance class	Selected phenotype	No. re-combinants studied	Relative frequency of unselected markers (%)				
					<i>proC</i>	<i>purE</i>	<i>gal</i>	<i>tol</i> ^b	<i>trp</i>
1	JF404-2a	G	Pur ⁺ Str ^r	476	57	100 ^c	39	17	10
2	JF404-6a	G	Pro ⁺ Str ^r	674	100	68	42	24	13
3	JF404-4a	F	Pur ⁺ Str ^r	678		100	46	24	9.9
4	JF404-11a	F	Pro ⁺ Str ^r	750	100	59	32	15	8.5
5	JF404-11a	F	Pur ⁺ Str ^r	520		100	51	24	10

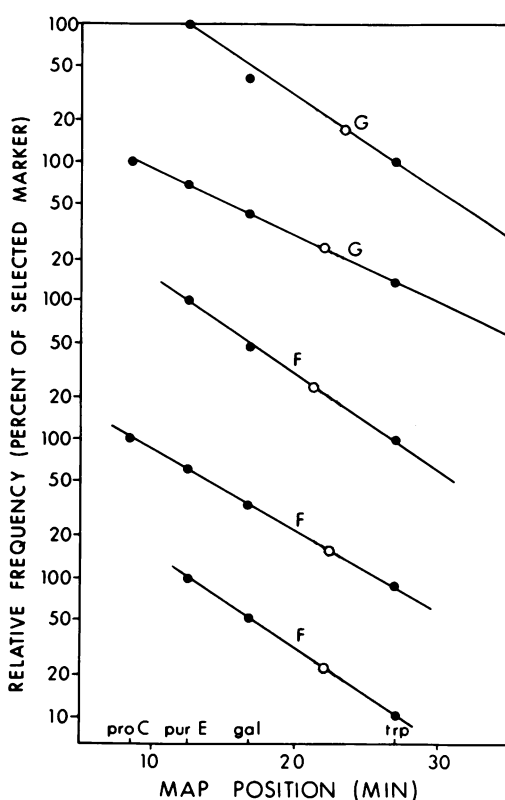
^a The recipient strain in each cross was CSH75.^b Tolerance was scored as resistance to bacteriocin JF246.^c The selected marker is given a value of 100%.

FIG. 1. Results of crosses presented in Table 7. The strains were mated using the gradient method and either *proC*⁺-recombinants or *purE*⁺-recombinants were tested for unselected markers indicated by the closed circles. The frequency of recombinants tolerant to bacteriocin JF246 was also tested and is indicated by the open circles. The location of the open circles on the gradient lines gives the approximate map position of the class F and class G mutations.

fraction of these may result in bacteriocin tolerance. We have isolated a large number of independent spontaneous mutants tolerant to a specific bacteriocin with the expectation that some of these mutants would exhibit alterations

in membrane components. The distinct patterns of sensitivity of the various tolerant mutants which we have studied to colicins, antibiotics, detergents, and chelating agents was not only useful in the phenotypic classification of mutants but may be a reflection of unique alterations in membrane components as well.

On the basis of the patterns of sensitivity to eight colicins, we grouped over 85% of our mutants into four major classes which we called classes A, B, F, and G. Each class had a distinct pattern of sensitivity when tested with a number of agents, such as antibiotics and detergents. A striking result of this classification was that over 70% of the mutants fell into one of two classes, class A and class B. These mutants were shown by complementation studies to be defective in the *tolA* or *tolB* genes. The preponderance of *tolA* and *tolB* mutants was found in several hundreds of spontaneous tolerant mutants isolated from three different parental strains. A similar preponderance of *tolA* and *tolB* mutants has been found by other workers who have isolated mutants tolerant to colicins A, E1, and/or K (14, 15).

We found *tolA* and *tolB* mutants were sensitive to the antibiotic bacitracin. Although we have no information to support the view, we believe it is likely that this sensitivity may be a reflection of an increased permeability of *tolA* and *tolB* cells to bacitracin.

Class F and class G mutants were distinguished from class A and class B mutants both phenotypically and genotypically. They represent new classes of colicin-tolerant mutants. Class G mutants differed from the parental type in that they were tolerant to bacteriocin JF246 and sensitive to EDTA and eosin yellow. Class F mutants differed from the parental strains in that they were tolerant to colicins A, K, and bacteriocin JF246 and sensitive to deoxycholate.

We propose to designate the genetic loci of the class F and G mutations tentatively as *tolF* and

tolG, respectively. They lie close to the map position of the tolerance loci called *tolD* and *tolE* (2, 5). A mutation in either *tolD* or *tolE* confers tolerance to colicins E2 and E3 as well as ampicillin resistance. *tolF* and *tolG* strains are sensitive to colicins E2 and E3.

Although the genetic loci which have been labeled *tolD*, *tolE*, *tolF*, and *tolG* may be distinct, since the phenotype of each class is distinct, further genetic tests are required to clarify this point.

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