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Interaction of Colicins with Bacterial Cells

III. Colicin-tolerant Mutations in *Escherichia coli*[1]

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Mutants that adsorb certain colicins without being killed, i.e., tolerant mutants (*tol*), were isolated from *Escherichia coli* K-12 strains. Selection was done either with colicin K or E2. Several groups of mutants showing different phenotypes were found, and some of them showed tolerance to both K and E colicins, which have different receptors. Many of these mutants mapped near *gal*. Typical mutants from group II, III, and IV were studied in more detail. The mutant loci were cotransducible with *gal* by phage P1. The linkage order was deduced to be *tol-gal*-λ. In partially diploid strains, these mutant loci are recessive to wild-type alleles. Temperature-dependent conditionally tolerant mutants were also isolated. Two groups were found: the first was tolerant to E2 and E3 at 40 C, but sensitive at 30 C; the second was tolerant to E2 at 30 C, but sensitive at 40 C. Experiments done with these mutants suggest that these mutations affect the heat lability of some protein that is necessary for the response of cells to colicins. Conditionally lethal tolerant mutants were isolated which at 40 C were tolerant to E2 and E3 and could not grow, but which at 30 C were fully sensitive and grew normally. The mutation mapped near *malA*. The tolerance at 40 C is not due to a consequence of an inactivation of general cellular metabolism, but presumably is a cause of the subsequent inhibition of cellular growth. The results suggest that some protein components involved in the response to colicin are also vital to normal cellular growth.

There are several distinctive features in the mode of action of colicins (Nomura, Ann. Rev. Microbiol., *in press*). First, the killing action of most colicins is a single-hit process as is the killing action of phage particles (11). Second, colicins do not penetrate bacteria but remain at the receptor on the bacterial surface and act from there (16, 18, 19). Third, different colicins exert different biochemical effects on sensitive cells (17, 18). Thus, colicin K-K235, like colicin E1-ML, inhibits deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis, but not respiration. Colicin E2-P9 specifically affects DNA synthesis and, in fact, causes DNA degradation, leading to subsequent inhibition of synthesis of other macromolecules. Colicin E3-CA38 inhibits protein synthesis, but not DNA or RNA synthesis, and causes specific inactivation of 30*S* ribosomal subunits (14). From the results of these studies, a general model for colicin action has been proposed (18). According to this model, each colicin has a "killing" or "biochemical" target, and the colicin stays at the receptor site and affects the target indirectly by a specific stimulus sent through a specific transmission system presumably located in the cytoplasmic membrane.

This model predicts several "steps" between adsorption and the final action on the target. Specifically, it predicts the existence of mutants which are resistant to colicin because of an alteration of some components involved in the proposed transmission system. Such mutants would adsorb a colicin but be resistant to its action. Thus, it is possible to distinguish two kinds of resistant mutants: one which has lost colicin receptors, and another which retains them but is still resistant to colicin action. This distinction was made independently by Nomura (18) and Clowes (5), who both isolated mutants of the predicted second type. Such mutants were previously called a "special class of resistant mutants" (18) or "mutationally immune" (5). To distinguish these mutants clearly from the first type, the mutants defective in the receptor, and from the colicinogenic cells which are resistant because of immunity conferred by the colicin factor, we shall designate them as colicin-"tolerant" mutants.

[1] Paper no. 1124 from the Laboratory of Genetics, University of Wisconsin, Madison.

The colicin-tolerant mutants may be further classified into three groups according to the model described above: those which fail to initiate the proposed stimulus at the receptor site, those which have some defect in the proposed transmission reaction, and those which have some alteration in the target itself. Mutation may cause these alterations either directly or indirectly. If the proposed "transmission system" (which includes components responsible for both initiation and transmission of the stimulus) involves some function which is essential for normal bacterial growth, some tolerant mutations may be deleterious or even lethal. Thus, study of tolerant mutations may be rewarding both in elucidating the mechanism of colicin action and in assessing the physiological significance of the proposed transmission system.

This paper consists of three major parts. The first part describes mutants of *Escherichia coli* K-12 isolated as tolerant to colicins E2 or K. Several different groups of mutants showing different phenotypes have been found and some of them have been located on the genetic map of the *E. coli* chromosome. Some of these mutants are similar to others studied by Reeves (20), Hill and Holland (*in preparation*), and Nagel de Zwaig and Luria (16*a*). The second part describes "conditionally tolerant" mutants which are tolerant to colicins at certain temperatures but not at others. Experiments suggest that these mutations affect the heat lability of some protein that is necessary for the response of cells to colicins. The final part of the paper describes "lethal colicin-tolerant" mutants, which at 40 C are tolerant to E2 and E3 but cannot grow, and which at 30 C are fully sensitive and grow normally. Experiments on these mutants have suggested that some protein components involved in the proposed transmission system are also vital to normal cellular growth.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains from which tolerant mutants were isolated, and other bacterial strains used, are listed in Table 1. For the sake of convenience, most of the tolerant mutants will be referred to by the mutant isolation number rather than by the strain number or by the group number in which the mutant is provisionally classified. All the mutants isolated by the use of colicin K as selective agent have

TABLE 1. *Characteristics of bacterial strains used*

Strain	Synonym or origin (obtained from)	Genetic characters or comments
Escherichia coli		
K-12 derivatives		
NO49	W3110Sm^r	F$^-$ *str-r*
NO443	(H. Ozeki)	F$^-$ *thr$^-$ leu$^-$ lac$^-$ gal$^-$ str-r*
AB1133	(A. Taylor)	*F$^-$ thr$^-$ leu$^-$ proA$^-$ his$^-$ arg$^-$ thi$^-$ lac$^-$ gal$^-$ xyl$^-$ ara$^-$ mtl$^-$ str-r* λ$^-$
NO523	AB1133	λ-Lysogenic derivatives of AB1133
HfrC	Hfr Cavalli	Hfr *met$^-$ str-s*
HfrH	Hfr Hayes	Hfr *str-s*
AB312	(A. Taylor)	Hfr *thr$^-$ leu$^-$ thi$^-$ lac$^-$ str-r*
AB313	(A. Taylor)	Hfr *thr$^-$ leu$^-$ thi$^-$ lac$^-$ str-r*
W4520	(H. Echols)	*met$^-$ gal$^+$* (λ)/F8-*gal$^+$*
S216	(E. R. Signer)	*thy$^-$ gal$^-$*/F1-*gal$^+$*
Colicinogenic strains		
NO39	*Salmonella typhimurium* (*cysD36 colE1-K30*) (H. Ozeki)	Produces colicin E1-K30
NO52	W3110Sm^r (*col*E2-P9)	Produces colicin E2-P9
CA38	*E. coli* CA38 (H. Ozeki)	Produces colicins E3-CA38 and I
NO38	*S. typhimurium* (*cysC7 str-r colK-K235*) (H. Ozeki)	Produces colicin K-K235
Lethal colicin-tolerant mutants[a]		
NO619	ER502-24, from AB1133	See text
NO620	ER502-34, from AB1133	See text
NO652	NO620	*thr$^+$ leu$^+$* derivative of NO620, obtained by conjugation

[a] Additional colicin-tolerant mutants are described in Table 2.

a prefix KR, and those isolated by E2 have a prefix ER.

Media. Tryptone broth, tris(hydroxymethyl)aminomethane (Tris) buffer-salt mixture, and Tris-glucose-Casamino Acids (TGC) were described previously (16). Nutrient agar plates, used for routine viable cell assays, contained 1.3% tryptone (Difco), 0.7% NaCl, 0.5% meat extract (Difco), and 1.2% agar (Difco). Synthetic complete agar plates (ACG) contained 0.063 M Na_2HPO_4, 0.04 M KH_2PO_4, 0.015 M $(NH_4)_2SO_4$, 8×10^{-4} M $MgSO_4$, 6×10^{-5} M $CaCl_2$, 2×10^{-6} M $FeCl_3$, 2% agar, 0.2% glucose, 2.5×10^{-5}% thiamine hydrochloride, and the following amino acids: L-threonine, L-leucine, L-histidine, and L-methionine, each 50 μg/ml; L-arginine, 200 μg/ml; L-proline, 20 μg/ml.

Synthetic sugar plates contained D-galactose (Difco), lactose (Fisher Scientific Co.), or D-xylose (Calbiochem), each 0.2% and replacing glucose in ACG plates. Eosin-methylene blue (EMB)-galactose plates contained (per liter): agar, 15 g; peptone (Difco), 10 g; galactose, 10 g; K_2HPO_4, 2 g; eosin Y, 0.4 g; methylene blue, 0.065 g. Both galactose and K_2HPO_4 were sterilized separately. EMB, EMB-lactose, and EMB-glucose plates were prepared in the same way, but galactose was omitted and lactose or glucose was used instead of galactose. Streptomycin was added to the plates at a concentration of 200 μg/ml, when counter-selection of streptomycin-sensitive cells was performed.

Colicin preparation. Colicin E2-P9 was prepared from an induced culture of strain NO52 [called *E. coli* W3110Sm^r (E2-P9) in our previous publications]. In most of the experiments, purified materials (16) were used. Colicins E3-CA38 and K-K235 were prepared and partially purified from induced cultures of *E. coli* CA38 and strain NO38, respectively as described previously (16). Colicin E1 was prepared in a similar way by inducing a culture of strain NO39 with mitomycin C. Killing activity of colicin I contained in the E3 preparation was about 0.1% of that of E3. The killing activity of colicins was assayed, and the activity was expressed as "killing units" as described previously (16). In this paper, colicins E1, E2, E3, and K refer to E1-K30, E2-P9, E3-CA38, and K-K235, respectively.

Isolation of colicin-tolerant mutants. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was done by a method similar to that described by Adelberg, Mandel, and Chen (1). Mutagen-treated cultures were grown overnight in tryptone broth and then treated with colicin E2 or K. Survivors were plated on nutrient agar plates. Resistant colonies were isolated and examined for their sensitivity to phages BF23 and T6. Phage BF23 shares the same receptor with E group colicins (6), and T6 with K (7). Mutants resistant to both E2 and BF23, or to both K and T6, were discarded as receptor-negative mutants. The remaining mutants were purified and examined for their sensitivity to other colicins. Mutants showing the same phenotype were kept only when they were of independent origin.

Isolation of lethal colicin-tolerant mutants. Mutagen-treated cells were grown at 30 C in tryptone broth to a titer of about 2×10^8/ml, transferred to 40 C, and treated with colicin E2 (about 10^{12} killing units/ml) for 30 min. After inactivation of the free as well as the adsorbed colicin with trypsin (250 μg/ml, 30 min), the culture was returned to 30 C, diluted with fresh tryptone broth, and incubated further. This process was repeated three times, and then the survivors were examined by replica-plating for their ability to grow at 40 C and for their colicin sensitivity at 30 C. Degradation of DNA by colicin E2 is very rapid at 40 C with the multiplicity used in the selection, and most of the cells are irreversibly killed before the addition of trypsin (*see* Table 6). Thus, most of the survivors must be resistant or tolerant to E2 at 40 C. Among survivors, mutant colonies which showed no growth at 40 C and were sensitive to E2 at 30 C were picked, purified, and examined for their colicin sensitivity at 40 C. Since these mutants do not grow at 40 C, they were grown at 30 C in tryptone broth and only the colicin sensitivity test was performed at 40 C. The test consisted of preincubation of cells at 40 C, treatment with E2 at 40 C, inactivation of E2 with trypsin at 40 C, dilution and plating, and counting the number of surviving colonies after overnight incubation of the plates at 30 C. By this test, seven temperature-sensitive mutants were found to be tolerant to E2 at 40 C but sensitive at 30 C. Two of them, ER502-24 and ER502-34, which are of independent origin, were studied further.

Linkage analysis by conjugation. Both Hfr and F$^-$ strains were grown in tryptone broth to a titer of about 2×10^8/ml. The two cultures were mixed in a ratio of about 1 Hfr to 9 F$^-$, and the mixture was incubated for 90 to 120 min without shaking or with gentle swirling. The mixture was then centrifuged, suspended in saline (0.85% NaCl), diluted, and plated on appropriate selective plates. Plates were usually incubated at 37 C (at 30 C in the case of temperature-sensitive mutants) for 40 hr. Recombinant colonies were picked, purified by restreaking on the selective plates employed, and examined for unselected markers. The colicin sensitivity test was done by cross-streaking the cells with the appropriate concentration of colicin on nutrient agar plates. Two control strains, sensitive (usually the Hfr parent) and tolerant (usually the F$^-$ parent), were always included on the same test plates.

P1 transduction. The procedure described by Adler and Kaiser (2) was followed. Transductants were purified by restreaking on the selective plates employed, and their characters were then examined.

Transduction of gal with λdg. The procedure described by Kaiser and Hogness (13) was followed. High-frequency-transducing lysates containing λ*dg* particles were prepared by mitomycin induction of heterogenote strains lysogenic for λ and λ*dg*. Three different λ*dg* phages were used. One (λ*dgA*) had its "left end" inside the galactokinase gene (3), and the other two (λ*dg71* and λ*dg126*) covered all the known sites in the *gal* operon.

Transfer of gal with F-gal episomes. Both donor strains (S216 or W4520), which are streptomycin-

sensitive (*str-s*), and the recipient strain (*str-r*) were grown in tryptone broth to a titer of about 2×10^8/ml. The two cultures were mixed at a ratio of about five donor cells to one recipient cell, and the mixture was incubated for 2 hr at 37 C without shaking. The culture was then diluted and plated on EMB-galactose plates containing streptomycin; *gal*$^+$ sexductants were isolated and purified by restreaking on EMB-galactose plates containing streptomycin.

Measurement of DNA degradation. Cells grown in TGC medium containing ^{14}C-uracil were used. Samples were treated with an equal volume of 1 N KOH and incubated at 37 C for 15 hr. Samples were then chilled to 0 C, neutralized with HCl, and treated with cold trichloroacetic acid (final concentration, 5%). Cell residues containing radioactive DNA were then filtered on membrane filters and washed with cold 5% trichloroacetic acid. The filters were glued on metal planchets, and their radioactivity was measured with a gas-flow counter.

Chemical determination of RNA and DNA. Cell suspensions were chilled to 0 C and then treated with cold trichloroacetic acid (final concentration, 5%). After 20 min at 0 C, samples were centrifuged and the pellets were washed once with cold 5% acid. The pellets were resuspended in 5% acid, and nucleic acids were then extracted by heating them in a boiling-water bath for 15 min. RNA in the extract was measured by the orcinol reaction (4) and DNA by the diphenylamine reaction (4).

RESULTS

Isolation and some properties of mutants tolerant to colicins. In our previous work (18), 46 tolerant mutants were isolated from the standard colicin-sensitive K-12 strain, NO49 (previously called W3110Sm^r), which had been used for our biochemical studies on colicin action. They were originally isolated by selecting for resistance to colicin E2. All of these 46 were shown to adsorb both colicin E2 and E3 and to be sensitive to phage BF23. These 46 mutants were classified into three main groups based on their sensitivity to other colicins: E1^s, E2^t, E3^t (sensitive to E1, but tolerant to E2 and E3), E1^s, E2^t, E3^s, and E1^t, E2^t, E3^t. Sensitivity was tested at 37 C. Only a few of the mutants were tested for sensitivity to colicin K. Because of the lack of suitable markers for genetic analyses, further studies of most of these tolerant mutants were abandoned. However, a few mutants showing temperature-dependent tolerance were studied biochemically (mutants ER437 and ER438, see below).

To obtain material for a genetic study of tolerance, isolation of mutants tolerant to E2 was repeated with several K-12 strains which had useful genetic markers. Altogether, 30 mutants of independent origin were obtained as tolerant mutants from strain NO523. They were classified into groups according to their pattern of sensitivity to colicins E1, E2, E3, and K. Three major classes of E2-tolerant mutants were obtained (Table 2). The first class of mutants (*tol II* in Table 2) is tolerant to all the E colicins as well as to colicin K. The other major classes are *tol III* (E1^s, E2^t, E3^t, K^t) and *tol IV* (E1^s, E2^t, E3^t, K^s). Colicin-tolerant mutants in general are referred to as *tol*, and the wild-type alleles, determining colicin sensitivity, are designated in general as *tol*$^+$. Various other types (*tol V* to *tol VII*, Table 2) were also found, but the number of mutants in each of these classes was small.

Colicin-tolerant mutants were also isolated by use of colicin K. Initially, mutants were isolated from strain NO443. Mutants tolerant to K (resistant to K, but sensitive to T6) were classified into two groups. The first class (*tol I* in Table 2) is sensitive to all of the E colicins. The second class is tolerant to all of the E colicins in addition to K, thus resembling the class *tol II* mutants selected with E2 from strain NO523. Therefore, the mutants in this class are classified in the same group (*tol II*) as the latter mutants. Mutants tolerant to K were also selected from strain AB1133 by use of colicin K. Three types, showing phenotypes corresponding to those of *tol I*, *tol II*, and *tol III*, were found.

The frequency of occurrence of a particular type of mutant differs greatly depending on the starting parental strain. As mentioned above, the type E1^s, E2^t, E3^s was of common occurrence in our previous work with strain NO49 as the parent, whereas only one mutant showing a similar pattern (*tol VII*) was found among 30 mutants from NO523. Similarly, mutants corresponding to *tol III* were not obtained from NO443, with colicin K as the selecting agent, but were quite common from AB1133.

As shown previously (18), these tolerant mutants do adsorb the colicins concerned. Several mutants, at least one from each of the four classes, *tol I* to *tol IV*, were tested for their adsorption of the colicin (K in the case of mutants selected by K, and E2 in the case of mutants selected by E2). The method used for this test was described previously (18). All of them adsorbed the colicin, but were resistant (tolerant) to it.

Clowes first found that mutants which are tolerant to E1 only [*tol VIII*, described by Nagel de Zwaig and Luria (16*a*)] are hypersensitive to methylene blue (5). All of the mutants listed in Table 2 were tested for their sensitivity to methylene blue, proflavine, and 2,4-dinitrophenol at several concentrations, and for their ability to grow on EMB and EMB-glucose plates. With a

TABLE 2. *Colicin-tolerant mutants isolated by selection with colicin K and colicin E2*

Class[a]	Resistance pattern[b]				Parent	Selection	No. isolated	Representative mutants studied in detail	No. linked to *gal*	No. not linked to *gal*
	E1	E2	E3	K						
tol I	s	s	s	t	NO443	K	6			
					AB1133	K	5	KR351	2	0
tol II	t	t	t	t	NO443	K	27	KR21, KR25	3	0
					AB1133	K	7			
					NO523	E2	5	ER102	3	0
tol III	s	t	t	t	AB1133	K	4			
					NO523	E2	5	ER72	4	0
tol IV	s	t	t	s	NO523	E2	15	ER50	12	3[e]
tol IVl[c]	s	s	s	s	AB1133	E2		ER502-24, ER502-34	0	1
tol IVt[c]	s	s	s	s	NO49	E2		ER437		
tol V	t	t	t	s	NO523	E2	3		1	2[e]
tol VI	t	t	s	s	NO523	E2	1		0	1[e]
tol VII	s	t	s	s	NO523	E2	1			
tol VIIt[d]	s	t	s	s	NO49	E2		ER438		
Total					NO443	K	33		3	0
					AB1133	K	16		2	0
					NO523	E2	30		20	6

[a] The designation of various classes of mutants are provisional names. The Roman numerals are used to designate specific patterns of colicin tolerance rather than specific cistrons. These names are adopted to agree with those used in the accompanying paper by Nagel de Zwaig and Luria (16*a*).

[b] Abbreviations: s, sensitive; t, tolerant.

[c] The resistance pattern shown was determined at 30 C; both *tol IVl* and *tol IVt* were tolerant to colicins E2 and E3 at 40 C.

[d] At 40 C, *tol VIIt* was sensitive to colicin E2.

[e] Results from both Hfr and F-*gal* conjugation experiments.

few exceptions, no pronounced differences were observed between these mutants and their respective parents.

Some colicin-tolerant mutants were found to be unstable, and during storage on agar slants gave rise to colicin-sensitive "revertants" with significant frequency. Such instability might suggest that the tolerant mutations in these mutants are harmful to the cell and therefore involve some components which are important in general cellular physiology. However, more definitive study on this point was done only on some tolerant mutants isolated in a different way. This will be described below.

Mapping of colicin-tolerant mutants. The mutants derived from strain NO443 (*tol I* and *tol II*) were crossed with HfrC: HfrC (*thr*$^+$ *leu*$^+$ *lac*$^+$ *gal*$^+$ *tol*$^+$ *str-s*) × F$^-$ (*thr*$^-$ *leu*$^-$ *lac*$^-$ *gal*$^-$ *tol str-r*). With the *tol II* mutants, analyses of *lac*$^+$ *str-r* and *gal*$^+$ *str-r* recombinants showed that tolerant mutant loci in this group of mutants are closely linked to the *gal* (90 to 100%), but not to the *lac*, locus. The close linkage to *gal* was also confirmed in crosses with another Hfr strain, HfrH. Mapping of the *tol I* mutants derived from NO443 has not been completed. Most of these mutants showed a greatly reduced frequency of recombination, and some difficulty was encountered in the genetic analysis by conjugation. However, two mutants, derived from another strain, AB1133, but which belong to the same class (*tol I*), were analyzed. Conjugation data showed that both are linked to *gal*, but not to *lac* or *his*. Data obtained with one of them, KR351, are shown in Table 3.

In the case of tolerant mutants selected with E2, only a few mutants were examined by Hfr

TABLE 3. *Frequency of unselected markers in the cross of a tol I mutant (KR351) with Hfr strains*[a]

Donor Hfr	Selected markers	Frequency of recombination	No. of recombinants tested	Per cent positive for unselected markers						Linkage of *tol I* to	
				thr$^+$ *leu*$^+$	*pro*$^+$	*lac*$^+$	*tol I*$^+$	*gal*$^+$	*his*$^+$	*gal*$^+$	*lac*$^+$
HfrH	*thr*$^+$ *leu*$^+$ *str-r*	1.8×10^{-2}	33	100	49	42	6	3	0	1/1	2/14
	pro$^+$ *str-r*	8.0×10^{-3}	36	56	100	61	14	14	0	5/5	5/22
	lac$^+$ *str-r*	8.0×10^{-3}	36	92	100	100	31	33	0	11/12	11/36
	gal$^+$ *str-r*	1.6×10^{-3}	36	94	100	100	100	100	0	36/36	36/36
	his$^+$ *str-r*	2.2×10^{-4}	35	31	26	23	12	12	100	4/4	3/8
HfrC	*lac*$^+$ *str-r*	5.2×10^{-2}	36	61	100	100	0	0	0		0/36
	pro$^+$ *str-r*	8.0×10^{-2}	36	22	100	92	0	0	0		0/33
	thr$^+$ *leu*$^+$ *str-r*	2.7×10^{-2}	36	100	78	81	0	0	0		0/29
	his$^+$ *str-r*	4.7×10^{-6}	36	3	0	6	0	0	100		0/2
	gal$^+$ *str-r*	$<1 \times 10^{-6}$	0								

[a] Colicin-tolerant mutant KR351 was F$^-$, *thr*$^-$, *leu*$^-$, *pro*$^-$, *lac*$^-$, *tol I*, *gal*$^-$, *his*$^-$, *str-r*. Both Hfr strains were Hfr, *thr*$^+$, *leu*$^+$, *pro*$^+$, *lac*$^+$, *tol I*$^+$, *gal*$^+$, *his*$^+$, *str-s*.

conjugation. Some tolerant mutants initially studied showed linkage to *gal*. This result, together with the linkage to *gal* of the *tol II* mutants selected with K, suggested a possible linkage to the same *gal* locus in many other mutants selected with E2. Therefore, all the mutants selected with E2 were first examined by sexduction with the F_1-*gal* episome. The F_1-*gal* episome carries a chromosome fragment that includes all of the *gal* genes and extends to the right of *gal* to cover the λ attachment site but not *pyrD*, and to the left of *gal* to cover the *su II*, *suB* genes but not *T6-s* (21). Mutants were mixed with strain S216 which carries the F_1-*gal*, and *gal*$^+$ sexductants were isolated. In most crosses (with 21 mutants), all the *gal*$^+$ sexductants were found to be colicin-sensitive (Table 2). However, *gal*$^+$ sexductants from six tolerant mutants remained colicin-tolerant (Table 2). These six mutants were further examined for the linkage of *tol* genes to *gal* by conjugation with HfrH or HfrC. In all, the absence of linkage to *gal* was confirmed. These six mutants were distributed into two minor classes (*tol V*, *tol VI*) and one major class (*tol IV*). Thus, of 26 tolerant mutants of independent origin selected by E2, all but 6 were found to be linked to *gal*. The presence of both *gal*-linked and unlinked mutants in the class *tol IV* shows that this class is not a homogeneous class of mutants.

Several mutants, which had been selected with K and found to be linked to *gal* by Hfr-conjugation [e.g., KR351 (*tol I*) and KR25 (*tol II*)], were also examined by sexduction with F_1-*gal*. All the *gal*$^+$ sexductants were colicin-sensitive. The results confirmed the linkage of these tolerance loci to *gal*.

It should be noted that the conversion to the colicin-sensitive phenotype by F_1-*gal* is not due to the presence of the F factor itself, nor due to the presence of *gal*$^+$ genes; *gal*$^+$ sexductants obtained from some of these mutants by use of another F-*gal*, F_8-*gal*, remained tolerant. F_8-*gal*, which is considerably shorter than F_1-*gal* (21), appears not to carry these tolerance genes.

Since the *gal*-linked *tol II*, *tol III*, and *tol IV* (Table 2) were major classes of tolerant mutants, a few representative mutants were selected from each of these groups and studied further.

Mutant loci in KR25, KR1, and KR21 (*tol II*), ER72 (*tol III*), and ER50 (*tol IV*) were all cotransduced with *gal* by phage P1 (Table 4). Linkage to *gal* was very high, ranging from about 60 to 100%. In these experiments, the presence or absence of the λ prophage was used as another unselected marker in addition to the *tol* gene. In the transduction experiments involving a λ$^+$ strain as the donor, preferential loss of λ$^+$ transductants might have taken place owing to possible induction of λ upon entry into λ$^-$ recipient cells. Nevertheless, with all the mutants examined, the results indicate that the order is not likely to be *gal*-λ-*tol*, but is either *gal*-*tol*-λ or *tol*-*gal*-λ. With mutants KR25 (*tol II*) and ER72 (*tol III*), the results are more consistent with the order *tol*-*gal*-λ than the order *gal*-*tol*-λ.

If *tol* mutation sites in these colicin-tolerant mutants were between *gal* and λ, these sites should be transducible with *gal* by λ*dg*. This possibility was examined. Colicin-tolerant mutants, KR21, KR25, ER50, and ER72, were treated with λ*dg*, and *gal*$^+$ transductants were isolated and examined for their sensitivity to the colicin. Three different λ*dg* phages were used. In all cases, *gal*$^+$ transductants were tolerant to the colicins, as were the original *gal*$^-$ tolerant mu-

TABLE 4. *Linkage of colicin tolerance loci (tol) to gal and λ loci studied by P1 transduction* [a]

Mutants		Recipient			Donor			Frequency of *gal*+ transductants scored as				Order found	Cotransduction frequency of *tol*+ with *gal*+, [(ABC + ABc)/total]
Name	Class	a	b	c	A	B	C	ABC	ABc	aBC	aBc		
KR25	*tol II*	*tol II*	*gal*−	λ−	*tol*+	*gal*+	λ+	2/88	50/88	6/88	30/88	(*tol II-gal*)-λ	0.59
KR1	*tol II*	*tol II*	*gal*−	λ−	*tol*+	*gal*+	λ+	6/90	83/90	0/90	1/90	(*tol II, gal*)-λ	0.99
KR21	*tol II*	*tol II*	*gal*−	λ−	*tol*+	*gal*+	λ+	0/89	58/89	1/89	30/89	(*tol II, gal*)-λ	0.65
ER72λ-*r*[b]	*tol III*	*tol III*	*gal*−	λ+	*tol*+	*gal*+	λ−	25/104	32/104	23/104	24/104	(*tol III-gal*)-λ	0.55
ER50λ-*r*[b]	*tol IV*	*tol IV*	*gal*−	λ+	*tol*+	*gal*+	λ−	21/79	50/79	0/79	8/79	(*tol IV, gal*)-λ	0.90

[a] P1 phages were grown in donor bacteria with the indicated genetic markers; *gal*+ transductants were examined for their sensitivity to colicin E2 and K (or E2 only in the case of ER50λ-*r*) as well as for the presence of λ. The presence or absence of λ was deduced from their capacity to allow T4*r*II growth; therefore, λ+ refers to the presence of the C_I gene in the λ prophage.

[b] ER72λ-*r* and ER50λ-*r* are λ-resistant derivatives of ER72 and ER50, respectively.

tants (Table 5). Since these tolerant mutation sites are recessive to wild-type alleles (*tol*+), as described in the next section, and since inhibition of *tol*+ gene function by neighboring λ genes is very unlikely, though not impossible, it was concluded that there is no cotransduction of these genes with *gal* by the λ*dg* used. Thus, the mutant loci in these tolerant mutants (KR21, KR25, ER50, and ER72) are not between *gal* and λ; hence, the order must be *tol-gal*-λ (*see* Fig. 9).

In most of these genetic analyses, transductants or recombinants were analyzed for their sensitivity to all of the colicins previously shown to be ineffective to the original *tol* mutants. With very few exceptions, cells which were shown to be sensitive to one colicin were always sensitive to all of the other colicins. Thus, the phenotype of mutants corresponding to *tol II*, *tol III*, and *tol IV* is not due to multiple mutations, but to a single mutation.

Mutants corresponding to *tol II*, *tol III*, and *tol IV* were also studied by other workers, and many of these mutations have been mapped near *gal* (16*a*, 20; Hill and Holland, *in preparation*). Some of these mutants tolerant to both K and E colicins may be identical to the class of mutants studied earlier (10). With *tol II* and *tol III* mutants, Nagel de Zwaig and Luria have shown that the order is *tol-gal-bio* in agreement with our results (16*a*). It should be noted also that the mutation to loss of E receptor was previously mapped between the *met* and *thi* loci (12, 20). The locus controlling the K receptor is the same as that of the T6 receptor and is also distinct from *gal*-linked colicin-tolerant loci (*see* Fig. 9).

Dominance of tol+ *over the tolerant mutation alleles.* As described above, 21 of 27 independent colicin-tolerant mutations in NO523 selected with E2 were shown to lie in the region covered by the F_1-*gal* episome. The sites of some mutations which were selected with K and classified as *tol I* or *tol II* were also shown to be in this region.

TABLE 5. *Absence of cotransduction of colicin tolerance genes with gal by Several λdg phages*

Recipient mutants (*gal*−)		Donor λdg	No. of *gal*+ transductants examined	No. of *gal*+ which were colicin-sensitive
Name	Class			
KR25	*tol II*	λdgA	31	0
		λdg71	7	0
		λdg126	5	0
KR21	*tol II*	λdgA	7	0
		λdg71	7	0
		λdg126	7	0
ER72	*tol III*	λdgA	2	0
		λdg71	2	0
		λdg126	2	0
ER50	*tol IV*	λdgA	3	0
		λdg71	3	0
		λdg126	3	0

The fact that, with these mutants, all the *gal*+ sexductants were sensitive to the colicin suggests that the wild-type (sensitive) alleles are dominant over mutant (tolerant) alleles. To establish this point, *gal*+ *tol*+ sexductants from KR25 (*tol II*), ER72 (*tol III*), and ER50 (*tol IV*) were examined for the segregation of *gal*− *tol* cells. In sexductants from ER72 and ER50, segregation of *gal*− cells was followed on EMB-galactose plates. Over 90% of the *gal*− cells segregated were shown to be colicin-tolerant, whereas all of the *gal*+ cells remained colicin-sensitive. This method could not be used with sexductants from KR25, since the original mutant KR25, like its parent NO443 (which both lack galactose-1-phosphate uridyl transferase), grew so poorly on EMB-galactose plates that *gal*− segregants were unlikely to form colonies on these plates. Therefore, cultures of a purified *gal*+ sexductant from KR25 were treated directly with colicin K and survivors were ex-

amined. As many as 2% of the cells survived as colony-formers, whereas fewer than 10^{-6} of the cells survived when *gal*$^+$ sexductants from the parent NO443 were treated with K under identical conditions. Moreover, 11 of 12 survivors from the *gal*$^+$ sexductant from KR25 were *gal*$^-$ and showed as poor growth on EMB-galactose plates as did the original KR25. Thus, the original *gal*$^+$ sexductants from these three mutants must have been a diploid with a structure *tol gal*$^-$/F$_1$-*tol*$^+$ *gal*$^+$. It was concluded that *tol II*$^+$, *tol III*$^+$, and *tol IV*$^+$ genes are dominant over the mutant alleles. Nagel de Zwaig and Luria have also shown that wild-type alleles are dominant over the *tol II* and *tol III* mutant alleles (16*a*).

Characterization of temperature-dependent tolerant mutants. The E2-tolerant mutants so far described were isolated at 37 C. Among the mutants isolated from the NO49 strain, some were found to be tolerant to the colicins only at certain temperatures. Two groups were found: the first, exemplified by ER437 (*tol IVt*), was resistant to E2 and E3 at 40 C, but sensitive at 30 C. The second, exemplified by ER438 (*tol VIIt*), was resistant to E2 (but not to E3) at 30 C, but sensitive to E2 at 40 C, though to a lesser extent than the parent.

Cells of ER437 were killed by colicin E2 or by E3 when grown and treated at 30 C, but were not killed when grown and treated at 40 C (Fig. 1). ER437 was sensitive to E1 at 30 C. Its sensitivity to E1 at 40 C is not clear, but is tentatively classified as sensitive. Although the mutant ER437 showed a considerable degree of resistance to E1 at 40 C, the parent NO49 also showed some resistance to E1 at 40 C, and the difference from the

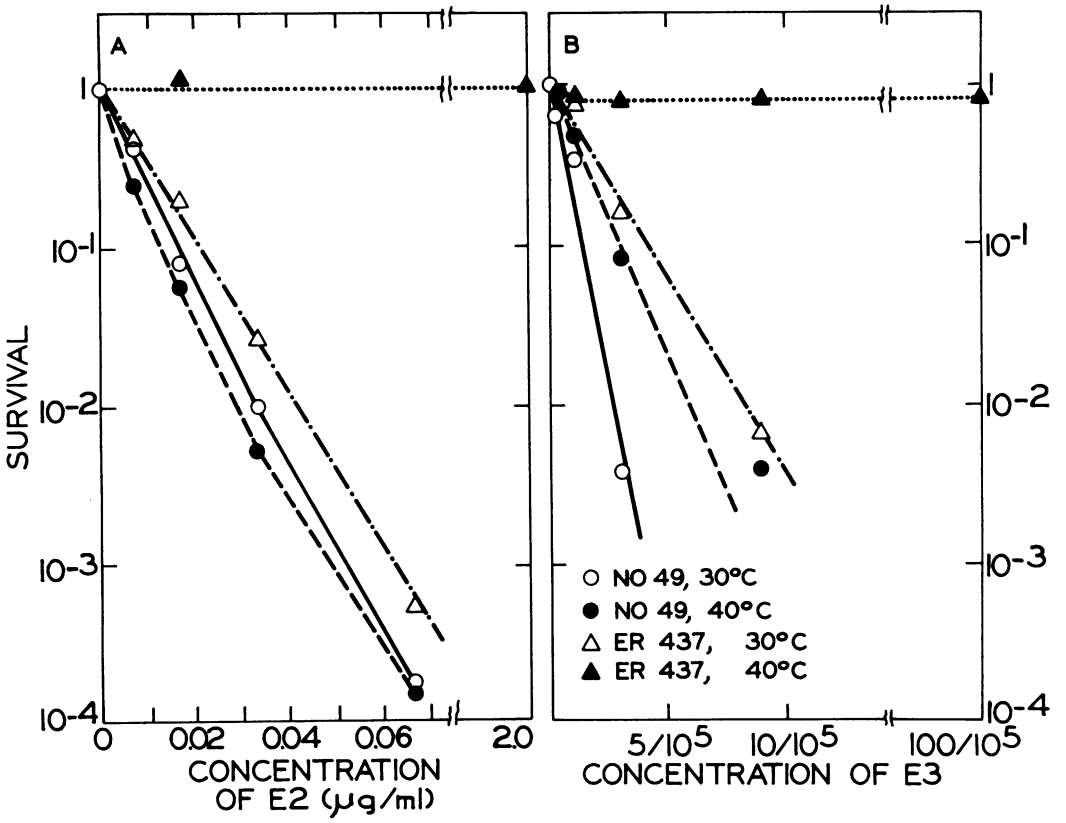


FIG. 1. *Survival curves for the conditionally tolerant mutant ER437 and its parent NO49 treated with colicin E2 (A) and E3 (B) at different temperatures. Cells were grown either at 30 or at 40 C in tryptone broth and then treated with various concentrations of colicins. After 30 min, samples were taken, diluted, and plated on nutrient agar plates. The plates for samples from the culture at 30 C were incubated at 30 C, and those from the culture at 40 C were incubated at 40 C. The fraction of survivors was calculated from the number of viable cells in the control tube at the end of the incubation time. E3 concentration is expressed as the final concentration of a particular E3 preparation used.*

mutant ER437 was not significant. The ER437 remained sensitive to colicin K both at 30 and at 40 C.

Cells of ER438 were killed by E2 when grown and treated at 40 C, but not when grown and treated at 30 C (Fig. 2). The killing at 40 C was not complete. The curve in Fig. 2 suggests that the culture contains a small population of cells with various degrees of resistance at 40 C. The response of ER438 to E1, E3, and K at 30 and 40 C was not significantly different from the parent NO49.

Both ER437 and ER438 are colicin-tolerant mutants, not receptor-negative mutants. Both are sensitive to phage BF23, which shares the receptor with E-group colicins. Mixing colicin E2 (or E3) with these mutants at the relevant temperatures (ER437 at 40 C and ER438 at 30 C) showed the disappearance of colicins from the solution. Moreover, the adsorption of purified radioactive colicin E2 (16) to ER437 cells was quantitatively compared with that of the parent at 40 C and no difference was observed. Thus, both ER437 and ER438 adsorb the colicin but are tolerant to the colicin at one temperature, whereas they are sensitive at another temperature. that is, they are conditionally tolerant to the colicin.

Conditional tolerance of the mutants ER437 and ER438 was also shown by biochemical methods. Colicin E2 causes DNA degradation (17). Therefore, the sensitivity of these tolerant mutants to colicin E2 was determined by following DNA breakdown. Colicin E2 caused extensive DNA degradation in the parent strain NO49 grown and treated at 30 and 40 C (Fig. 3). ER437 behaved like the parent at 30 C, but did not show any recognizable DNA degradation when grown and treated with E2 at 40 C. On the other hand, ER438 behaved like the parent when grown and tested at 40 C, but was resistant to E2-induced DNA degradation when grown and tested at 30 C.

The mutant ER437, the first type, showed weak growth in tryptone broth at 40 C, but normal growth at 30 C. It also gave only small colonies on nutrient agar plates at 40 C after about 40 hr of incubation, but gave the parental-size colonies after overnight incubation at 30 C. The mutant ER438, the second type, showed no apparent defect in its growth characteristics at either 30 or 40 C.

Some preliminary experiments suggested that the observed poor growth of ER437 at 40 C is a consequence of the mutation to colicin tolerance. However, the mutant ER437 did not have useful genetic markers to do genetic studies, and, therefore, the relationship between the temperature-sensitive growth characteristics and the temperature-dependent tolerance was not studied further. With other conditionally tolerant mutants to be described in later sections, this relationship was studied.

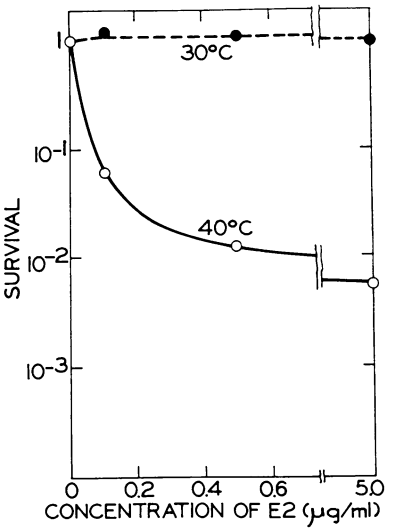


FIG. 2. *Survival curves for the conditionally tolerant mutant ER438 treated with colicin E2 at different temperatures. The procedure was similar to that described in Fig. 1.*

Mechanism of temperature-dependent tolerance. ER437 showed tolerance at 40 C to both E2 and E3, which have different biochemical targets. Therefore, the altered component is not related to the target, but is related to other steps in the proposed transmission system. To assess the nature of the altered component, a few experiments were performed.

ER437 can take two different states: the colicin E2 (and E3)-sensitive state at 30 C and the colicin E2 (and E3)-tolerant state at 40 C. Using colicin E2-induced DNA degradation as a criterion of sensitivity to E2, we studied the conditions necessary for the changes from the sensitive to the tolerant state, or from the tolerant to the sensitive state. As described above (Fig. 3 and 4A), ER437 cells grown at 30 C are sensitive to E2 and show DNA degradation upon E2 treatment at 30 C. Such sensitive cells were preincubated at 40 C for 30 min under conditions of no cellular growth [e.g., in the absence of a nitrogen source or in the presence of chloramphenicol (CM)], and then sensitivity to E2 was tested at 30 C. No DNA degradation was observed (Fig. 4B). Thus, the conversion from the sensitive to the tolerant

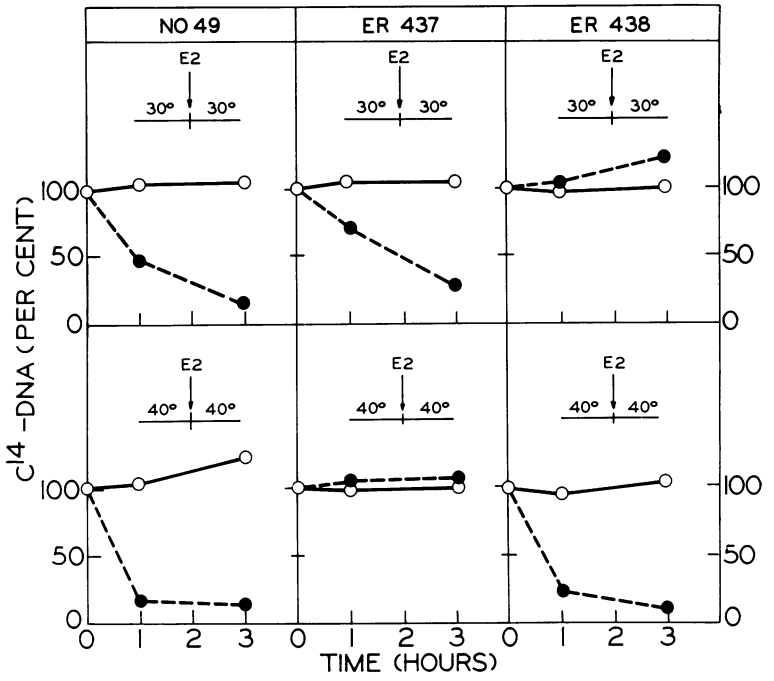


FIG. 3. *Effect of temperature on the DNA degradation induced by colicin E2. Parent strain NO49 and conditionally tolerant mutant strains ER437 and ER438 were grown in TGC medium containing ^{14}C-uracil either at 30 or at 40 C. Cells were harvested, washed, and resuspended in TGC medium containing ^{12}C-uracil (50 μg/ml). Cells grown at 30 C were treated with E2 (about 2 × 10^{12} killing units/ml) at 30 C, and cells grown at 40 C were treated with the same concentration of E2 at 40 C. Radioactivity in the DNA fraction per milliliter of culture was measured after incubation for 0, 1, and 3 hr, and is expressed as a percentage of the amount of radioactivity at time zero.* (○) *Untreated cells;* (●) *colicin-treated cells.*

state takes place by heating cells at 40 C for 30 min, and does not require protein synthesis. However, heating of washed cells in a buffer-salt solution at 40 C for 30 min converted cells from the sensitive state to an only weakly tolerant state. Addition of an energy source, such as glucose, was necessary for the efficient conversion from the sensitive to the tolerant state. However, more studies are needed before we establish the minimal requirements for this conversion and assess the mechanism involved.

Conversion of ER437 cells from the tolerant to the sensitive state was also studied. As shown before (Fig. 3), cells grown at 40 C were tolerant to DNA degradation induced by E2 at 40 C. Such tolerant cells grown at 40 C were transferred to 30 C, incubated for 1 hr under growth conditions, and then treated with E2 in the presence of CM. DNA degradation was observed (Fig. 4D) as in the cells initially grown at 30 C (Fig. 4A). However, when the preincubation at 30 C was done in the presence of CM, no significant DNA degradation was observed (Fig. 4C). Thus, CM inhibits the conversion from the tolerant state to the sensitive state after the temperature shift-down. These results suggest that cells grown at 40 C lack an active component whose synthesis is CM-sensitive. Therefore, the mutationally altered component is presumably a protein necessary for the response of cells to colicins. The mutation makes this protein heat-labile.

Similar studies were done with the second type of temperature-dependent tolerant mutant, ER-438. The conversion from the tolerant state (produced in cells grown at 30 C) to the sensitive state took place after incubation at 40 C for 30 min in the absence of protein synthesis (Fig. 5A, 5B). The conversion from the sensitive state (cells grown at 40 C) to the tolerant state also took place in the absence of protein synthesis (in the presence of CM), though less efficiently, after preincubating the cells at 30 C for 30 min (Fig. 5D). The simplest interpretation is that the altered component is a protein which is necessary for the colicin action and which can take a "functional" configuration at 40 C but not at 30 C. However, other explanations are also possible. Whatever the altered component of ER438 is, it

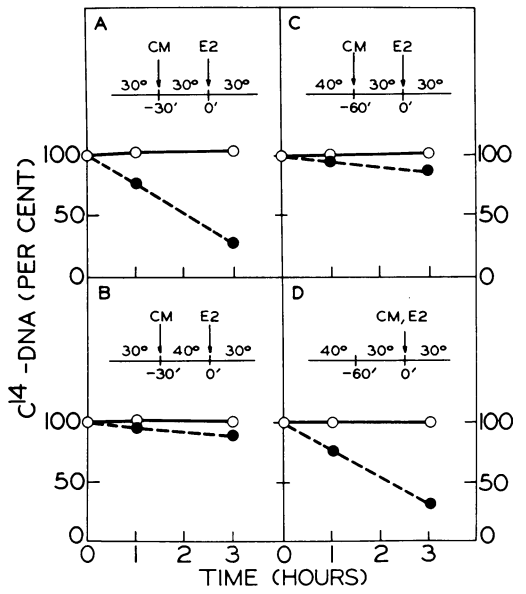


FIG. 4. *Effect of temperature on E2-induced DNA degradation in the conditionally tolerant mutant ER437. Cells were grown in TGC medium containing ^{14}C-uracil either at 30 C (A and B) or at 40 C (C and D). Cells were harvested, washed, and resuspended in TGC medium containing ^{12}C-uracil. Cells were then preincubated either at 30 C (A, C, D) or at 40 C (B), in the presence or absence of chloramphenicol (CM) as indicated. Cells were then treated with E2 (2 × 10^{12} killing units/ml) and incubated at 30 C. DNA degradation was followed. (○) Untreated cells; (●) colicin-treated cells.*

may be different from the temperature-sensitive component in ER437. ER438 is tolerant to E2 and not to E3 at 30 C, whereas ER437 shows the temperature-dependent tolerance both to E2 and to E3.

Mapping of mutant loci in these mutants has not been done. However, the properties of ER437 are very similar to those of the lethal colicin-tolerant mutant ER502-34 described below.

Lethal colicin-tolerant mutations. Mutants were isolated, which at 40 C were tolerant to E2 and E3 and could not grow, but which at 30 C were fully sensitive and grew normally. Two of these lethal tolerant mutants, ER502-24 and ER502-34, of independent origin, were studied in detail.

Mutants ER502-24 and ER502-34 adsorbed E2 but were much more resistant to E2 at 40 C than was the parent strain AB1133 (Table 6). These mutant cells were treated with E2 at 40 C for 40 min; a part of the culture was then diluted to prevent any further adsorption of the colicin, and the number of survivors was assayed at 30 C. Another part was treated with trypsin at 40 C for an additional 30 min, and then the number of survivors was assayed at 30 C. Colicin was adsorbed by mutant cells at 40 C, but cells were not irreversibly killed at this temperature; that is, the cells were in a "trypsin-reversible state." Cells without trypsin treatment had adsorbed colicin by the time of dilution, and the irreversible killing took place during the subsequent incubation on the assay plates at the lower temperature. After trypsin treatment, most, or a significant fraction, of the E2-treated cells were recovered as colony-formers. The number of survivors recovered after trypsin treatment in these experiments varied between 10 and 100% (Table 6). In contrast, most of the parent AB1133 cells were irreversibly killed before the addition of trypsin, and the number of colony-formers recovered after trypsin treatment was smaller than that of the tolerant mutants by at least a factor of 100.

Both ER502-24 and ER502-34 were found to be tolerant to colicin E3 as well as to E2 at 40 C, but were sensitive to them at 30 C. Conditional tolerance of these mutants to the colicins was also

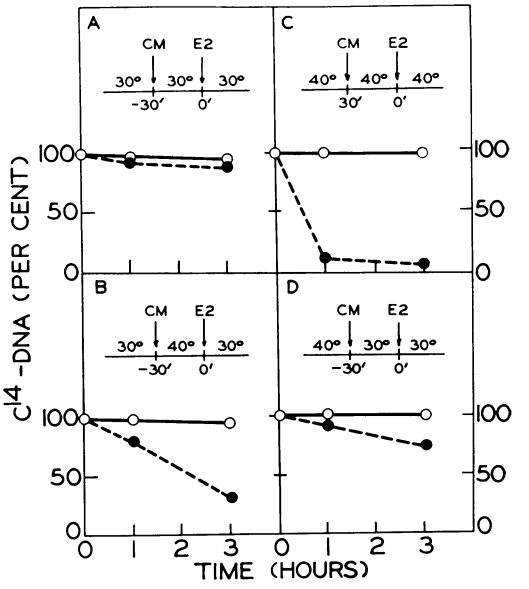


FIG. 5. *Effect of temperature on E2-induced DNA degradation in the conditionally tolerant mutant ER438. Cells of the mutant ER438 were grown in TGC medium containing ^{14}C-uracil either at 30 C (A and B) or at 40 C (C and D). Cells were harvested, washed, and resuspended in TGC medium containing ^{12}C-uracil. Cells were then preincubated for 30 min either at 30 or at 40 C as indicated, and then treated with E2 (about 2 × 10^{12} killing units/ml) at 30 C. DNA degradation was followed. (○) Untreated cells; (●) colicin-treated cells.*

shown by biochemical methods. As described before for the mutant ER437, the sensitivity to E2 was measured by following DNA degradation. Transfer of the mutant ER502-34 cells to 40 C converted cells to a state resistant (tolerant) to E2-induced DNA degradation (Fig. 6). The same conclusion was also obtained by measuring inhibition of RNA or DNA synthesis by colicin E2 after the temperature shift from 30 to 40 C (cf. Table 8). Colicin E3 specifically inhibits protein synthesis (14, 18). The sensitivity to E3 was measured by following ^{14}C-amino acid incorporation into proteins after the treatment of cells with E3. Mutant ER502-34 was sensitive to

TABLE 6. *Killing action of colicin E2 on temperature-sensitive, colicin-tolerant mutants ER502-24 and ER502-34, and on temperature-resistant transductants derived from them*[a]

Expt	Strain	Survivors (%)	
		At 40 min (no trypsin)	At 70 min (trypsin added at 40 min)
1	AB1133 (parent)	0.0045	0.025
	ER502-24 (mutant)	0.012	65
	ER502-24-T16 (transductant)	<0.001	0.6
	ER502-34 (mutant)	0.06	66
	ER502-34-T15 (transductant)	0.001	0.55
2	AB1133 (parent)	0.001	0.044
	ER502-34 (mutant)	0.004	14
	ER502-34-T1 (transductant)	0.002	0.060

[a] Cells were grown in TB medium at 30 C to a titer of about 2 × 10^8/ml, harvested, washed, and suspended in Tris buffer-salt solution at the original concentration. Cell suspensions were incubated at 40 C for 30 min, and then treated (time zero) with colicin E2 (about 10^{12} killing units/ml). At 40 min, samples were taken and the number of colony-formers was assayed. Trypsin (final concentration, 250 μg/ml) was added to the remaining part, and the incubation was continued for an additional 30 min. The number of survivors was then assayed. Control cells without E2 treatment were also incubated. No significant change in the number of survivors in the controls was observed. Temperature-resistant transductants were isolated from temperature-sensitive, colicin-tolerant mutants by use of phage P1, and their sensitivity to colicin E2 was compared with that of mutants and parent strains.

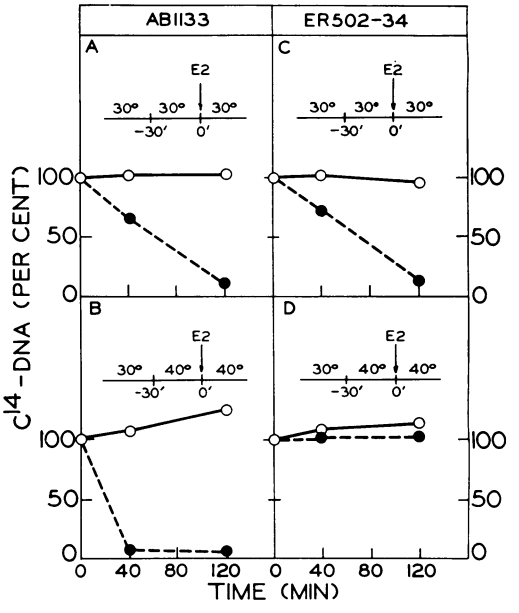


FIG. 6. *Effect of temperature on E2-induced DNA degradation in the lethal colicin-tolerant mutant ER502-34 and its parent AB1133. Cells were grown in TGC medium containing ^{14}C-uracil at 30 C to a density of about 2 × 10^8/ml at 30 C. Cells were harvested, washed, and resuspended in TGC medium containing ^{12}C-uracil. They were preincubated for 30 min either at 30 C (A, C) or at 40 C (B, D), and then treated with E2 (2 × 10^{11} killing units/ml) and incubated at the indicated temperatures. DNA degradation was followed.* (○) *Untreated cells;* (●) *colicin-treated cells.*

E3 at 30 C, but became resistant (tolerant) to E3 after transfer to 40 C (Fig. 7). Treatment at 40 C for 30 min was sufficient to convert cells to the tolerant state; this state was retained for some time (about 30 min), even after transfer of the cells back to 30 C. As expected, continued growth at 30 C brought cells back to the original sensitive state so that the inhibition by E3 became eventually apparent. The same conclusion was also obtained with respect to the sensitivity to E2.

Preliminary biochemical experiments showed that conditions necessary for the conversion of ER502-34 or ER502-24 cells from the sensitive to the tolerant state, or vice versa, are similar to those found with the mutant ER437.

Genetic correspondence between temperature sensitivity and temperature-dependent colicin tolerance. As mentioned before, mutants ER502-24 and ER502-34 are temperature-sensitive. They cannot form colonies on solid media at 40 C, but can form colonies at 30 C. The question whether the two characteristics, temperature sensitivity and conditional tolerance, are due to

the same mutation was studied. Mutant cells were treated with phage P1 which had been grown on the parent strain AB1133. The transductants which grew at 40 C on solid media were isolated and tested for their sensitivity to colicin E2 at 40 C. As shown in Table 7, 149 transductants from ER502-34 and 49 transductants from ER502-24 were analyzed, and all of them were found to be sensitive to E2 at 40 C (*see also* Table 6).

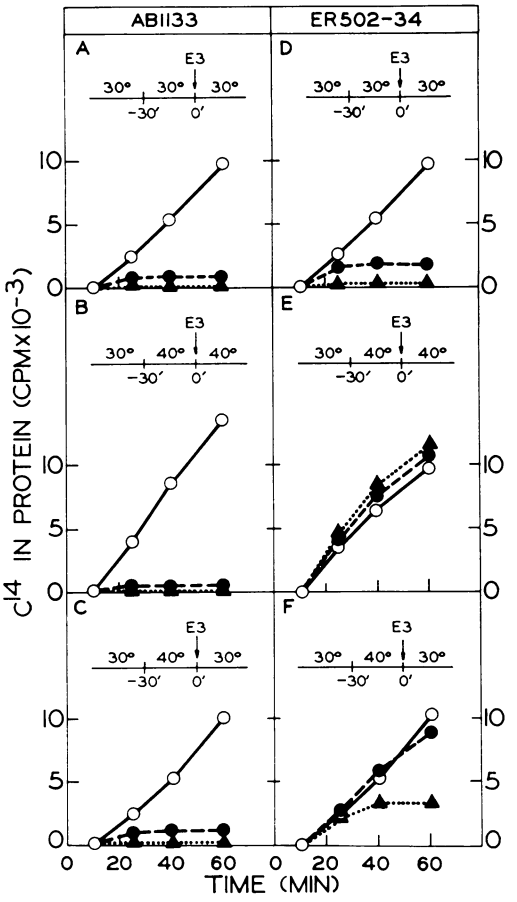


FIG. 7. *Effect of temperature on the inhibition of protein synthesis by E3 in the lethal colicin-tolerant mutant ER502-34 and its parent AB1133. Cells were grown at 30 C in TGC medium supplemented with arginine (200 μg/ml), threonine, leucine, histidine, proline (each 50 μg/ml), vitamin B_1 (5 μg/ml), and Casamino Acids (5 μg/ml instead of 100 μg/ml). When the cell densities reached about 2 × 10⁸/ml, the cultures were divided and incubated either at 30 or at 40 C for 30 min as indicated. Cells were then treated with E3 (about 2 × 10⁹ killing units/ml, ●; or about 10¹⁰ killing units/ml, ▲); 10 min later, ^{14}C-phenylalanine (about 2 mc/mmole, 5 μg/ml) was added, and ^{14}C incorporated into the protein fraction was followed both in the control (○) and in the E3-treated cultures.*

Temperature-resistant recombinants obtained from crosses between the mutant ER502-34 and several Hfr strains were also analyzed. Altogether, 92 recombinants were analyzed, and all of them were found to be sensitive to E2 at 40 C (Table 7).

In similar experiments, temperature-resistant transductants and exconjugants were analyzed for their sensitivity to E3 as well as to E2 at 40 C. All were sensitive to both E2 and E3 at 40 C.

Analysis of temperature-resistant transductants or exconjugants for their tolerance to colicin was also done by a biochemical method. Both types of temperature-resistant recombinants were sensitive to E2 at 40 C, whereas recombinants from the same conjugation experiment but which had not received the temperature-resistant gene remained tolerant at 40 C (Table 8).

Finally, spontaneous temperature-resistant "revertants" were examined. Altogether, 11 revertants, which grew at 40 C both on solid and in liquid media, were analyzed for their sensitivity to E2 at 40 C. All of them were sensitive to E2.

These results show that temperature sensitivity and tolerance to E2 and E3 at 40 C are most likely due to a single mutation. This suggests that the same mutationally altered component is essential both for the response to colicin action and for cellular growth.

Mapping of the "lethal colicin-tolerant" locus. The mutation in ER502-34 occurs in what we shall tentatively call the "lethal colicin-tolerant" locus (*tol IVl*). The locus *tol IVl* was mapped on the *E. coli* chromosome by bacterial crosses.

A *thr*$^+$ *leu*$^+$ derivative of mutant ER502-34, NO652, was used as recipient, and two Hfr strains, AB313 and AB312, were used as donors.

AB313 injects its chromosome with *xyl* as a leading marker and in the order *xyl-his-gal-proA-leu-thr* (22; Fig. 9). From analysis of unselected markers in the cross AB313 × NO652 (Table 9), it was concluded that the *tol IVl* locus is between *xyl* and *his*. Among recombinants selected for *his*$^+$ *thr*$^+$ *leu*$^+$ in this cross, the type *xyl*$^-$ *his*$^+$ was examined. The recombinants of this type must have had a crossover between *xyl* and *his* (Table 10). If it is assumed that relative frequency of crossovers between two markers is proportional to the distance between them, the data in Table 10 can be used to estimate the approximate map position of *tol IVl*. The distance between *xyl* and *tol IVl* is thus very roughly 10% of the distance (about 30 min) between *xyl* and *his*, or 3 min.

AB312 injects its chromosome with *malA* as a leading marker and in the order *malA-xyl-thr-leu-proA* (22; Fig. 9). The data in Table 11 suggest

that the *tol IVl*$^+$ locus is injected as one of the early markers, between the chromosomal origin of AB312 and *xyl*. Analysis of recombinants belonging to the class *tol IVl*$^+$ *thr*$^+$ *leu*$^+$ *proA*$^+$ showed that the distance between *tol IVl* and *xyl* is approximately one-third of the distance (about 19 min) between *xyl* and *thr*, or about 6 min (Table 12).

TABLE 7. *Genetic correspondence between temperature sensitivity and temperature-dependent colicin E2 tolerance in mutants ER502-24 and ER502-34*[a]

Expt	Type of expt	Donor	Recipient	Selection	Temperature-resistant transductants (or recombinants)		
					No. analyzed	Tolerance to E2 and E3 at 40 C	
						Tolerant	Sensitive
1	P1 transduction	AB1133 (*tol IVl*$^+$)	ER502-34 (*tol IVl*)	Temp resistance	149	0	149
2		AB1133 (*tol IVl*$^+$)	ER502-24 (*tol IVl*)	Temp resistance	49	0	49
3	Conjugation	HfrH (*tol IVl*$^+$ *str-s*)	ER502-34 (*tol IVl str-r*)	Temp resistance and *str-r*	45	0	45
4		HfrC (*tol IVl*$^+$ *str-s*)	ER502-34 (*tol IVl str-r*)	Temp resistance and *str-r*	47	0	47

[a] Temperature-resistant transductants or exconjugants were isolated, purified, and tested for their tolerance to E2 at 40 C by use of a method similar to that described in Table 6. Control tolerant mutants (ER502-34 or ER502-24) showed between 10 and 100% survivors after E2 and trypsin treatment. The plates showing survivors comparable to the parent AB1133 strain (less than 1% of the survivors of control tolerant mutants) were scored to indicate that the test strain is sensitive to E2 at 40 C.

TABLE 8. *Genetic correspondence between temperature sensitivity and temperature-dependent colicin E2 tolerance as determined by a biochemical method*[a]

Expt	Type of expt	Strains (no. analyzed)	Inhibition of ^{14}C-adenine incorporation by E2 at 40 C
			%
1	P1 transduction (selected for temperature resistance)	AB1133	96
		ER502-34	0
		Temperature-resistant transductants (3)	96, 90, 88
2	Conjugation: AB312 × NO652 (ER502-34 *thr*$^+$ *leu*$^+$) (selected for *xyl*$^+$ *thr*$^+$ *leu*$^+$)	AB1133	91
		NO652	12
		Recombinants	
		xyl$^+$ *thr*$^+$ *leu*$^+$ *tol IVl*$^+$ (5)	91, 96, 96, 97, 94
		xyl$^+$ *thr*$^+$ *leu*$^+$ *tol IVl* (5)	6, 10, 4, 20, 0

[a] In experiment 1, temperature-resistant P1 transductants were isolated, purified, and analyzed for their tolerance to E2 at 40 C (*see* below). In experiment 2, a *thr*$^+$ *leu*$^+$ derivative of tolerant mutant ER-502-34, NO652, was crossed with AB312, and *xyl*$^+$ *thr*$^+$ *leu*$^+$ recombinants were selected (*see* Table 12). These recombinants were then analyzed for their temperature sensitivity; 58% were temperature-sensitive (*tol IVl*), like the original temperature-sensitive female strain, NO652, and 42% were found to be temperature-resistant (*tol IVl*$^+$), like the male AB312. Five of each recombinant type were arbitrarily selected, and their tolerance to E2 at 40 C was examined in the following way. Cells were grown in TGC medium at 30 C to a titer of about 2 × 10^8/ml, and were then transferred to a water bath (40 C). After 30 min at 40 C, colicin E2 (about 10^{12} killing units/ml) was added (time zero). At 10 min, ^{14}C-adenine (0.7 mc/mmole, 7 μg/ml) was added; the incorporation was stopped at 30 min by cooling the cultures to 0 C. The amount of ^{14}C-adenine incorporated into the total nucleic acid fraction (cold 5% trichloroacetic acid-precipitable materials) was measured and compared to the control incorporation in the absence of E2. The degree of inhibition of the incorporation by colicin E2 was calculated.

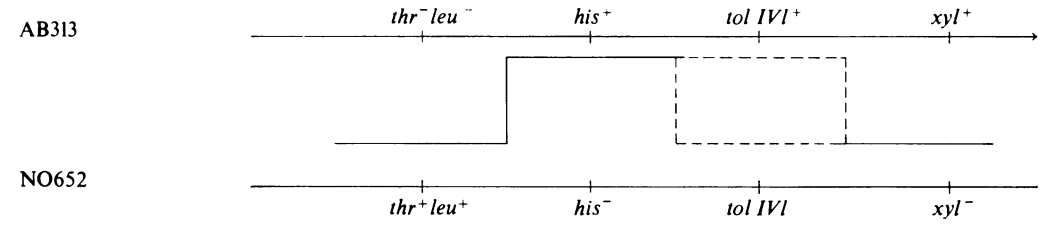
TABLE 9. *Frequencies of unselected markers in the cross AB313 × NO652 (ER502-34-thr⁺ leu⁺)*[a]

Selected markers	Frequency of recombination	No. of recombinants tested	Per cent positive for unselected markers			
			xyl⁺	*tol IVl⁺*	*his⁺*	*proA⁺*
xyl⁺ thr⁺ leu⁺	2.2×10^{-3}	60	100	5	0	0
tol IVl⁺ thr⁺ leu⁺	9.0×10^{-4}	56	68	100	2	0
his⁺ thr⁺ leu⁺	2.3×10^{-4}	96	25	33	100	0
proA⁺ thr⁺ leu⁺	4.3×10^{-5}	36	3	0	3	100

[a] AB313: Hfr, *xyl⁺*, *tol IVl⁺*, *his⁺*, *proA⁺*, *thr⁻*, *leu⁻*; NO652: F⁻, *xyl⁻*, *tol IVl*, *his⁻*, *proA⁻*, *thr⁺ leu⁺*.

TABLE 10. *Frequency of crossovers between xyl and tol IVl and between tol IVl and his*[a]

Cross	Type of recombinants	Total no. examined	No. found		Relative frequency of crossovers between	
			tol IVl⁺	*tol IVl*	*xyl* and *tol IVl*	*tol IVl* and *his*
AB313 × NO652 (ER502-34-*thr⁺leu⁺*)	*xyl⁻ his⁺*	82	7	75	7	75



[a] The *his⁺ thr⁺ leu⁺* recombinants were selected. Among these recombinants, the type *xyl⁻ his⁺* was examined. Data from two experiments are combined.

These results show that the *tol IVl* locus is between the AB312 chromosomal origin and *xyl*, and the distance from *xyl* is approximately 3 to 6 min. The results suggest that the locus is in the neighborhood of the *malA* or *str* locus (Fig. 9). Preliminary experiments showed that the locus is in fact linked to *malA* more closely than to *xyl*.

Growth characteristics of the lethal colicin-tolerant mutant ER502-34 at 40 C. To obtain some hints concerning the biochemical basis of the temperature sensitivity of the mutant ER502-34 at 40 C, macromolecular synthesis in the mutants was analyzed at 40 C. Cells were first grown at 30 C in the TGC medium and then transferred to 40 C. As shown in Fig. 8, turbidity of the culture continued to increase (but at a decreasing rate) for about 2 hr and reached a value about three times the initial value. Protein synthesis was also followed and the results were similar. DNA synthesis also continued for 2 to 3 hr, and the amount of DNA reached a value three to four times higher than the initial value. Cells continued to divide for 2 to 3 hr, and the number of viable cells increased by a factor of four to five. The only significant early defect found was RNA synthesis. The amount of RNA increased by about 50 to 80% in 1 hr, and then the increase stopped. Synthesis of RNA and DNA was also followed by measuring incorporation of ^{14}C-uracil into the RNA and DNA fractions. Essentially the same conclusion was obtained; the incorporation of ^{14}C-uracil into RNA at 40 C ceased about 90 min after the transfer of cells from 30 to 40 C, whereas the incorporation into

TABLE 11. *Frequency of unselected markers in the cross AB312 × NO652 (ER502-34-thr⁺ leu⁺)*[a]

Selected markers	Frequency of recombination	No. of recombinants tested	Per cent positive for unselected marker		
			tol IVl⁺	*xyl⁺*	*proA⁺*
tol IVl⁺ thr⁺ leu⁺	9×10^{-3}	48	100	10	0
xyl⁺ thr⁺ leu⁺	8×10^{-3}	48	25	100	0
proA⁺ thr⁺ leu⁺	3×10^{-4}	48	33	31	100

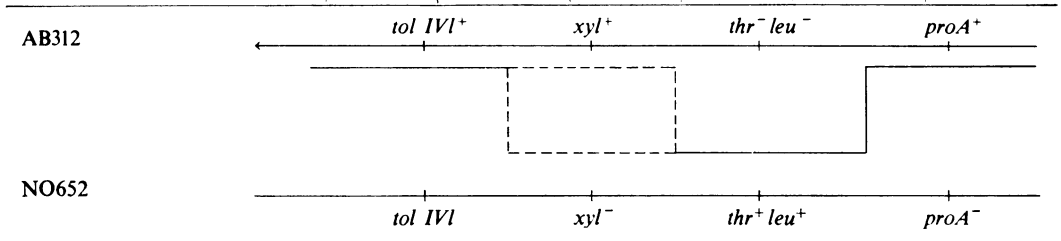
[a] AB312: Hfr, *tol IVl⁺*, *xyl⁺*, *thr⁻*, *leu⁻*, *proA⁺*; NO652: *tol IVl*, *xyl⁻*, *thr⁺*, *leu⁺*, *proA⁻*.

DNA continued for a longer time. Somehow, synthesis of RNA was affected by the temperature shift earlier than other macromolecular synthesis.

It should be noted, however, that the conversion of mutant cells from the colicin-sensitive state to the tolerant state takes place before significant inhibition of macromolecular synthesis, including RNA synthesis. The conversion is

TABLE 12. *Frequency of crossovers between tol IVl and xyl, and between xyl and thr leu*[a]

Cross	Expt	Total no. examined	No. found		Relative frequency of crossovers between	
			xyl⁻	*xyl*⁺	*tol IVl* and *xyl*	*xyl* and *thr leu*
AB312 × NO652 (ER502-34-*thr*⁺*leu*⁺)	1	20	6	14		
	2	16	4	12		
	3	36	8	28		
	Total	72	18	54	18	54



[a] In experiments 1 and 2, *proA*⁺ *thr*⁺ *leu*⁺ recombinants were selected. Among these recombinants, the type *tol IVl*⁺ *thr*⁺ *leu*⁺ was analyzed. In experiment 3, *tol IVl*⁺ *thr*⁺ *leu*⁺ *proA*⁺ recombinants were directly selected, and analyzed for the *xyl* character.

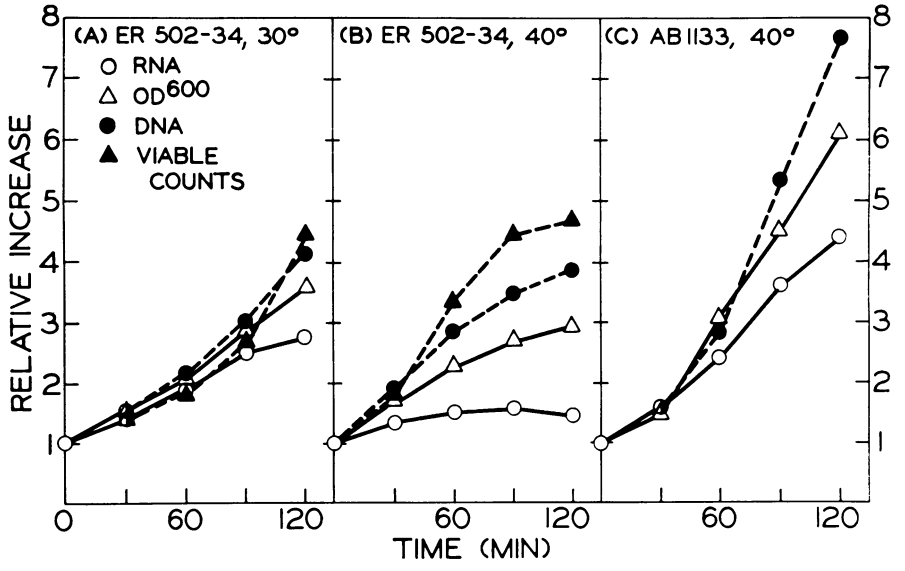


FIG. 8. *Growth characteristics of the lethal colicin-tolerant mutant ER502-34. Cells of the mutant ER502-34 were grown in TGC medium at 30 C to a density of about 2 × 10⁸/ml. The culture was divided into two parts, and one was incubated at 30 C (A) and the other at 40 C (B). Samples were taken at the indicated times, and the amount of DNA and RNA was analyzed chemically. The turbidity of the culture (optical density at 600 mμ) and the number of viable cells were also determined. Plates for viable-cell counts were incubated at 30 C. In a similar experiment, growth characteristics of the mutant ER502-34 after transfer to 40 C were compared with those of the parent AB1133 under the same conditions. ER502-34 gave results very similar to those in Fig. 8B. The results obtained with the parent AB1133 are shown in Fig. 8C.*

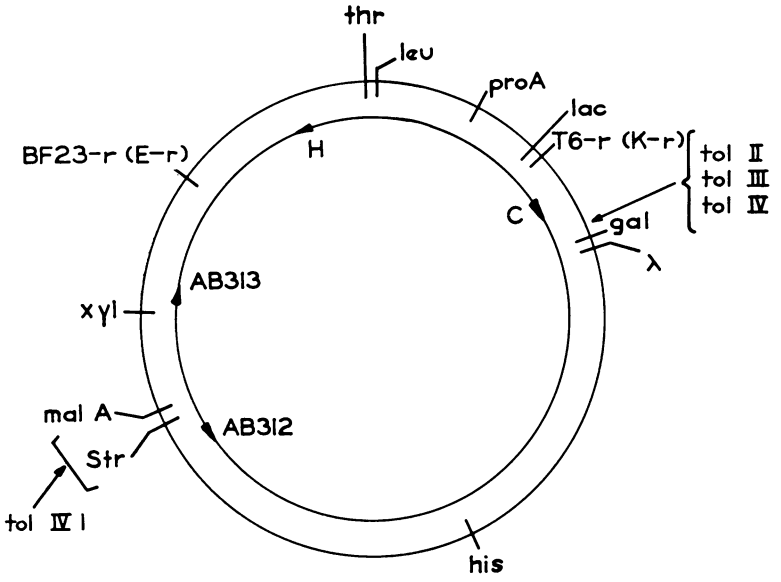


FIG. 9. *Genetic map of Escherichia coli K-12 showing loci used in recombination experiments and several colicin-tolerant and lethal colicin-tolerant loci. K-r is the locus controlling the colicin K receptor and is the same as that of the phage T6 receptor (T6-r). Similarly, E-r is the locus controlling the colicin E receptor and is the same as that of the phage BF23 receptor (BF23-r).*

usually complete within 30 min after the transfer (e.g., Fig. 7). Simple temperature shock (40 C, 30 min) does not affect subsequent RNA synthesis at 30 C, but the shocked bacteria retain the acquired colicin tolerance for about 30 min or longer during subsequent growth at 30 C. It appears that the alteration at 40 C which is responsible for the colicin tolerance is specific, and is not a consequence of, but presumably a cause of, the subsequent inhibition of macromolecular syntheses.

DISCUSSION

As discussed in the introduction, colicin-tolerant mutants may comprise several different types. According to the possible "step" affected, mutants can be classified into three groups: those which fail to initiate the proposed stimulus at the receptor site, those which fail to transmit this stimulus to the target, and those which have some alteration in the target and fail to respond to this stimulus.

In addition to possible mutations which affect these steps directly, there may be many other mutations which affect these steps indirectly, thus increasing the degree of complexity greatly. This anticipated complexity has in fact been encountered. As shown in Table 2, simple selection of tolerant mutants with E2 revealed several different kinds of tolerant mutants. [These complex tolerance patterns make the possibility unlikely that "tolerance" in general involves the release of proteases which destroy colicins. Such a possibility has also been excluded by experiments with temperature-dependent tolerant mutants; mutants which were tolerant at 40 C for several hours were subsequently killed after dilution and plating at 30 C. This experiment shows that colicins are adsorbed and remain intact at the receptor site while cells are in a tolerant state. In addition, adsorption experiments done with several tolerant mutants and with radioactive colicins failed to reveal any protease activity in the mutants.] Similar complex patterns were also observed by other workers (6*a*, 20; Hill and Holland, *in preparation*).

The first feature observed is that many tolerant mutants selected with E2 or with K and showing several different phenotypes are clustered close to the *gal* operon on the genetic map of the *E. coli* K-12 chromosome. Some are tolerant only to some E-group colicins and others are tolerant only to K, whereas many others are tolerant both to K and E colicins. It would be interesting to determine how many cistrons are involved in this region, and what kind of functional relationship, if any, exists among several different kinds of mutant loci clustered in this region. From complementation analysis, Nagel de Zwaig and Luria showed that mutations *tol II* and *tol III* involve at least two different cistrons (16*a*).

Colicin K and some of the E-group colicins have different receptors and affect different targets. Yet, many isolated mutants are tolerant to

both K and E-group colicins (*tol II* and *tol III*). The mutant genes may be concerned with synthesis of proteins which themselves are a common structural element in some step of the transmission systems both for colicin K and for some of the E-group colicins. Alternatively, the mutant genes may be concerned with synthesis of enzymes which are involved in the synthesis of some common chemical components necessary for some step in each of these transmission systems. Although the second possibility seems to be more likely, the distinction must await future studies.

Unlike certain tolerant mutations discussed above (*tol II* and *tol III*), which might have indirect effects on colicin sensitivity, the mutations in temperature-dependent tolerant mutants (*tol IVt*, *tol VIIt*, and *tol IVl*) are very likely in genes which control proteins more intimately involved in some step of the proposed transmission reaction. Analyses of conditions necessary for conversion of the tolerant state to the sensitive state, and vice versa, with mutants ER437 (*tol IVt*) and ER502-34 (*tol IVl*) have shown that the mutationally altered component is probably a protein which becomes heat-labile, and that this protein must be present continuously in an active form in order to keep cells in the sensitive state. The biochemical targets for colicin E2 and E3 are different. The fact that ER437 and ER502-34 are tolerant to both E2 and E3 simultaneously at 40 C shows that the altered component is not related to the target, eliminating one possibility described above. Distinction between the other possibilities, i.e., failure to initiate the "stimulus" at the receptor site, and failure to transmit the "stimulus," may be difficult at the present moment. In any event, our results strongly indicate that there is a protein which is directly involved in the specific response of cells to colicin E2 and E3, but which is not essential for adsorption of these colicins. The mutant locus in ER502-34, *tol IVl*, which is concerned with the synthesis of this protein, has been mapped on the *E. coli* K-12 chromosome at a site distinct from both *gal*-linked tolerant loci and the E-receptor locus (Fig. 9).

As discussed previously (18), the transmission system is presumably located in the cytoplasmic membrane. Hence, some of the tolerant mutants, being defective in the transmission directly or indirectly, may show some alteration in the cytoplasmic membrane. Nagel de Zwaig and Luria have studied properties of colicin-tolerant mutants similar to our *tol II* and *tol III* mutants, as well as mutants tolerant to E1 only (*tol VIII*). The properties observed have been interpreted by them as being due to abnormalities in the cytoplasmic membrane (16*a*). The heat-labile proteins in ER437 or ER502-34, which are directly involved in the transmission system as discussed above, may also be located in the cytoplasmic membrane.

With most of the tolerant mutants linked to *gal* (*tol II*, *tol III*, and *tol IV*), it was shown that the tolerant mutations are recessive to the standard sensitive alleles. This indicates that the mutations cause loss of some function which the wild-type alleles can supply in mero-diploid strains. If products of the mutant *tol* genes exist, the presence of these gene products in mero-diploid strains would not interfere with the function of the wild-type gene product. Such analysis has not been done with temperature-dependent tolerant mutants, *tol IVt* and *tol VIIt*, and lethal tolerant mutants, *tol IVl*.

Resemblance of the tolerance to immunity was pointed out previously (5, 18). Colicinogenic immune cells adsorb homologous colicins (16), but are resistant to them (8). However, there may be some significant differences between the mechanism of tolerance and that of immunity. Immunity is probably due to the synthesis of a specific substance (an "immunity substance") which interferes with the proposed transmission mechanism (17) and is a dominant character. Nevertheless, as discussed previously (18), both immunity and tolerance may involve alterations in components of the cellular membrane.

Finally, we have shown that some of the tolerant mutations are lethal. Mutants ER502-34 and ER502-24, discussed above, at 40 C are tolerant to E2 and E3 but cannot grow; at 30 C, they are fully colicin-sensitive and grow normally. Various genetic experiments indicated that the tolerance at 40 C and the temperature sensitivity of growth are due to a single mutation. Moreover, the tolerance is not a consequence of inhibition of macromolecular synthesis or of energy-supplying reactions, but presumably is a cause of the subsequent inhibition of cellular growth. It should also be emphasized that the present lethal tolerant mutations in these mutants involve temperature-sensitive proteins. Our experiments show that such protein components involved in the transmission system are also vital to normal cellular growth. One could speculate about various possible roles of such a protein in normal cellular physiology. Some of the possible functional roles of membranes and membrane proteins involved in the response to colicins were previously discussed in relation to regulation and cellular organization of bacterial cells (15, 18; Nomura, Ann. Rev. Microbiol., *in press*). It is hoped that further physiological studies of the various types of tolerant and lethal tolerant mutants as well as identification and study of altered components in these tolerant mutants may con-

tribute to elucidation of the mechanism involved in the response of the bacterial cells to colicins, and also to our understanding of the functions of cellular membranes in various biological systems.

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LITERATURE CITED

1. ADELBERG, E. A., M. MANDEL, AND G. C. C. CHEN. 1965. Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. ADLER, J., AND A. D. KAISER. 1963. Mapping of the galactose genes of *Escherichia coli* by transduction with phage P1. Virology **19**:117–126.
3. ADLER, J., AND B. TEMPLETON. 1963. The amount of galactose genetic material in λ*dg* bacteriophage with different densities. J. Mol. Biol. **7**:710–720.
4. ASHWELL, G. 1957. Colorimetric analysis of sugars, p. 73–105. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.
5. CLOWES, R. C. 1965. Transmission and elimination of colicin factors and some aspects of immunity to colicin E1 in *Escherichia coli*. Zentr. Bakteriol. Parasitenk. Abt. I. Orig. **196**:152–159.
6. FREDERICQ, P. 1949. Sur la resistance croisée entre colicine E et bacteriophage II. Compt. Rend. Soc. Biol. **143**:1011–1013.
7. FREDERICQ, P. 1949. Sur la resistance croisée entre colicine K et bacteriophage III. Compt. Rend. Soc. Biol. **143**:1014–1016.
8. FREDERICQ, P. 1956. Resistance et immunité aux colicines. Compt. Rend. Soc. Biol. **150**:1514–1517.
9. FREDERICQ, P. 1958. Colicins and colicinogenic factors. Symp. Soc. Exptl. Biol. **12**:104–122.
10. GRATIA, J. P. 1964. Resistance à la colicine B chez *E. coli*. Relations de specificité entre colicines B, I et V et phage T1. Ann. Inst. Pasteur **107**:132–151.
11. JACOB, F., L. SIMINOVITCH, AND E. L. WOLLMAN. 1952. Sur la biosynthèse d'une colicine et sur son mode d'action. Ann. Inst. Pasteur **83**: 295–315.
12. JENKIN, C. R., AND D. ROWLEY. 1955. Resistance to colicin E as a genetic marker in *E. coli* K12. Nature **175**:779.
13. KAISER, A. D. AND D. S. HOGNESS. 1960. The transformation of *Escherichia coli* with deoxyribonucleic acid isolated from bacteriophage λ*dg*. J. Mol. Biol. **2**:392–415.
14. KONISKY, J., AND M. NOMURA. 1967. Interaction of colicins with bacterial cells. II. Specific alteration of *Escherichia coli* ribosomes induced by colicin E3 *in vivo*. J. Mol. Biol. **26**:181–195.
15. LURIA, S. E. 1964. On the mechanisms of action of colicins. Ann. Inst. Pasteur **107**:67–73.
16. MAEDA, A., AND M. NOMURA. 1966. Interaction of colicins with bacterial cells. I. Studies with radioactive colicins. J. Bacteriol. **91**:685–694.
16*a*. NAGEL DE ZWAIG, R., AND S. E. LURIA. 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J. Bacteriol. **94**: 1112–1123.
17. NOMURA, M. 1963. Mode of action of colicines. Cold Spring Harbor Symp. Quant. Biol. **28**: 315–324.
18. NOMURA, M. 1964. Mechanism of action of colicines. Proc. Natl. Acad. Sci. U.S. **52**:1514–1521.
19. NOMURA, M., AND M. NAKAMURA. 1962. Reversibility of inhibition of nucleic acids and protein synthesis by colicin K. Biochem. Biophys. Res. Commun. **7**:306–309.
20. REEVES, P. 1966. Mutants resistant to colicin CA42-E2: cross resistance and genetic mapping of a special class of mutants. Australian J. Exptl. Biol. Med. Sci. **44**:301–316.
21. SIGNER, E. R., J. R. BECKWITH, AND S. BRENNER. 1965. Mapping of suppressor loci in *Escherichia coli*. J. Mol. Biol. **14**:153–163.
22. TAYLOR, A. L., AND M. S. THOMAN. 1964. The genetic map of *Escherichia coli* K-12. Genetics **50**:659–677.