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Isolation and Characterization of an *Escherichia coli* Mutant Tolerant to Colicins Ia and Ib

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Two classes of spontaneous colicin I insensitive mutants of *Escherichia coli* have been isolated. The first class (called *cir*) has lost its ability to adsorb either colicin Ia or Ib, maps at 41 min on the *E. coli* genetic map, and retains sensitivity to all other colicins tested. The *cir* phenotype is probably due to an alteration in the colicin I receptor. The second class of mutant (called *tolI*) retains full capacity to adsorb [^{125}I]colicin I and, therefore, represents the isolation of a mutant tolerant to colicin I. The *tolI* mutant is sensitive to all other colicins tested and has a map location of 89-1 min. The *tolI* mutant grows with a reduced mass yield when glucose is used as a carbon source and cannot utilize succinate or acetate for growth. The *tolI* mutant shows a reduced sensitivity to sodium azide and phenethylalcohol. It is suggested that *tolI* is deficient in some aspect of aerobic metabolism which must be operative for colicin I sensitivity.

The selection of *Escherichia coli* strains insensitive to the action of a particular colicin has led to the isolation of two mutant classes—resistant and tolerant (23). Colicin-resistant mutants are defective in their capacity to adsorb colicin. Although it has been possible to solubilize the cell envelopes of *E. coli* in such a way as to release the protein receptors of colicin E3 and K (26), colicin M (2), and colicin Ia and Ib (18), no receptor activity can be solubilized from the corresponding resistant strain.

Colicin-tolerant mutants, on the other hand, retain a capacity to adsorb colicin, even though the mutant can survive colicin treatment. It should be pointed out, however, that in no case has the binding of a particular colicin to its cognate-tolerant strain been characterized in sufficient detail to eliminate the possibility of an altered colicin receptor. However, such an interpretation is unlikely for several colicin-tolerant mutants. For example, mutants of tolerant class *tolA* are insensitive to colicins E1, E2, E3, K, and A (22, 25), even though, of these colicins, only E2 and E3 share a common receptor. Likewise *tolB* mutants are tolerant to colicins A, E2, E3, and K (22, 25).

Based on the sensitivity of *tolA* and *tolB* mutants to a variety of agents, it has been suggested that the pleiotropic properties of these mutants are derived from a change in cell surface structure (1, 22). In the case of *tolC* which is tolerant to colicin E1 alone, the mutation was interpreted as leading to a change in the property of the cytoplasmic membrane (1, 22, 30). A change in cell surface structure may also be responsible for the phenotypes of *tolD* (3) and *E* (8), tolerant to colicins E2 and E3, *tolF* (12), tolerant to bacteriocin bc246 and colicin A, and *tolG* (12), tolerant to bacteriocin 246, and *cet* (28), tolerant to colicin E2. In each of these classes it is possible that the tolerant mutation leads to a reduction in the probability that the colicin can interact with its primary cell target.

Colicins Ia and Ib are structurally related colicins (15) with a mode of action showing similarities to colicins E1, A, K, and bc246. Both colicins inhibit macromolecular synthesis (19) and affect the activity of several transport systems (M. J. Gilchrist and J. Konisky, manuscript in preparation). In this investigation, we describe the isolation and characterization of a new class of tolerant mutant selected for insensitivity to colicin Ib. The mutant is physiologically and genetically distinct from other isolated *tol* mutants and is uniquely tolerant to colicins Ia and Ib. We propose to name this mutant class *tolI*.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used are described in Table 1. TB (tryptone, Difco) medium and plates were prepared as previously described (19). Medium 63 (20) broth and Davis

TABLE 1. *Bacterial strains*[a]

Strain	Genotype and comments	Source
JK 114	*E. coli* K-12 DG78-X36, F⁻, *argH, ara, lac, gal, ura, trp, his, thi, mal man, xyl, purC, tonA, str-r.*	P. Carl
JK 89	*E. coli* Hfr strain KL 16 *thi* λ⁻	S. Kaplan
JK 16	*E. coli* K-12 W3110 λ⁻ (Col Ia-CA53), source of colicin Ia	
JK 20	*E. coli* K-12 W3110 λ⁻ (Col Ib-P9), source of colicin Ib	
JK 46	*S. typhimurium* cys 7, str-r (Col K-K 235), source of colicin K	M. Nomura
JK 71	*E. coli* K-12 R2.1/V^{r}λ^r, source of colicin B.	P. Fredericq
JK 138	*E. coli* K-12 AB1867 *thi, thr, leu, lac, str-r, azi, supE.*	B. Bachmann
JK 139	*E. coli* K-12 AB253 *thi, thr, leu, lac, str-r, supE.*	B. Bachmann

[a] The nomenclature is that recommended by Taylor and Trotter (25).

minimal broth and plates (6) were supplemented with the appropriate growth requirements. In all cases growth was at 37 C.

Preparation and assay of colicins. Purified colicins Ia and Ib were prepared as previously described (15). Iodination of purified colicin Ib (16) led to radioactive derivatives, exhibiting 75 to 100% of the original biological activity observed with noniodinated colicin. For quantitation of specific activity, the colicin was assumed to have a molecular weight of 80,000 (15). Purified colicins E2 and E3 were obtained from M. Nomura, and purified colicin E1 was obtained from D. Helinski. For the preparation of colicin K and B, an overnight culture of the appropriate colicinogenic strain was sonicated for 1 min and then centrifuged at 80,000 × *g* for 1.5 h. The supernatant fraction was used directly. Sensitivity of strains to these colicins was determined by the end point dilution method (19). In all cases the assay was carried out in triplicate.

Isolation of resistant and tolerant mutants. Independent spontaneous mutants were isolated by growing a small inoculum (20 cells) of the parental strain (JK 114) in TB broth for about 18 h. The culture was then cross-streaked versus colicin Ib and TB agar plates. After 24 h of incubation, isolated colonies appearing in the zone of inhibition were isolated, and their insensitivity to colicin Ib and retention of parental markers were verified.

Genetic procedures. Mapping by the gradient of transmission method (6) was carried out as described by Miller (20). Hfr (JK 89) × F⁻ matings (ratio 1:10) were carried out at 37 C in TB broth for 100 min before being diluted and plated on appropriate selective Davis minimal media. Recombinant colonies were picked, purified by restreaking on the selective plates employed, and examined for unselected markers. Sensitivity to colicin Ib was tested by cross-streaking the cells with colicin on TB plates. In all matings, the validity of the gradient mapping method was verified by a determination of the absolute number of recombinants for each marker. In all cases, a plot of the log of the number of recombinants versus map position gave a straight line.

RESULTS

Pattern of sensitivity. Two independent and spontaneous colicin Ib insensitive mutants were isolated from *E. coli* K-12 strain JK 114. When the sensitivity of each strain to a variety of colicins was tested, the results shown in Table 2 were obtained. As can be seen, the mutant strain JK 114R1 shows a reduced sensitivity to both colicins Ia and Ib when compared to the parental strain (JK 114). This is in contrast to the mutant JK 114R3, which is totally insensitive to Ia and Ib. Neither mutant has lost sensitivity to colicins E1, E2, E3, K, or B (Table 2). Furthermore, JK 114R1 is fully sensitive to colicin A, colicin C, and bacteriocin bc246 (J. Foulds, personal communication). The differential sensitivity of the mutants to colicin Ib was also demonstrable by analysis of survival curves. At a colicin Ib concentration leading to less than 1% survivors of the parental strain, insensitive strains JK 114R1 and JK 114R3 exhibited 30 and 11% survivors, respectively.

Colicin adsorption. The capacity of the colicin Ib-insensitive mutants to adsorb colicin Ib was determined by direct measurement of cell-bound [^{125}I]colicin Ib (Fig. 2). Whereas mutant strain JK 114R1 shows normal binding when compared to the parental strain, strain JK 114R3 has lost most of its capacity for colicin Ib adsorption. Similar results are seen with [^{125}I]-colicin Ia. Scatchard plots of similar data show

TABLE 2. *Sensitivity of parent and insensitive mutants to various colicins*

Colicin	Colicin sensitivity[a]		
	JK 114	JK 114R1	JK 114R3
Ia	1,280	160	<1
Ib	640	160	<1
E1	3,200	3,200	3,200
E2	3,200	3,200	3,200
E3	1,600	1,600	1,600
K	8	8	8
B	8	8	8

[a] The data are expressed as the reciprocal of the maximum colicin dilution still inhibiting cell growth.

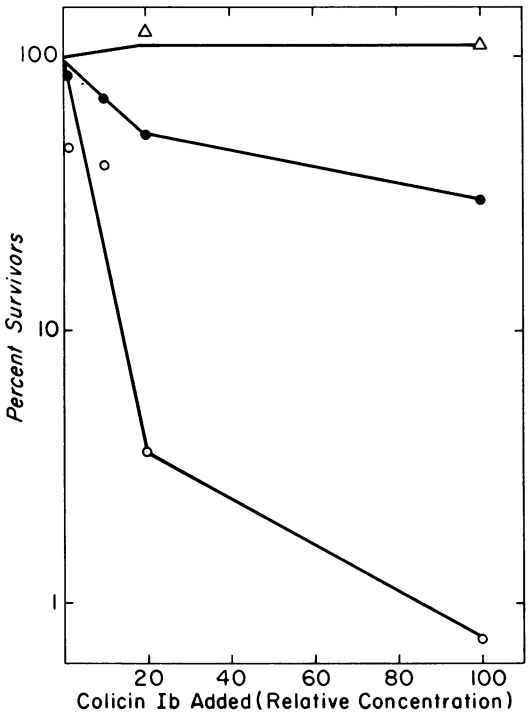

FIG. 1. *Survival curve of colicin Ib-treated parental strain JK 114 and two colicin I insensitive mutants JK 114R1 and JK 114R3. Mixtures containing 0.4 ml of bacterial cultures (2 × 10⁸ to 4 × 10⁸ cells per ml) grown in TB broth and 0.1 ml of various colicin dilutions were incubated for 20 min at 37 C before being diluted and plated for colony counts on TB agar plates. The percentage of survivors was calculated from the number of viable cells in the control tube (no colicin) at the end of the incubation time. Strain JK 114, O; strain JK 114R1, ●; strain JK 114R3, Δ.*

that strain JK 114 and JK 114R1 have approximately 720 and 640 colicin Ib receptors, respectively. The association constant for the Ib-receptor interaction is approximately 4.8×10^{11} M^{-1} at 37 C for both. Based on these experiments, strains JK 114R1 and JK 114R3 are classified as tolerant and resistant mutants, respectively. A pattern of colicin sensitivity exhibited by JK 114R1, has not been previously reported. Thus, this mutant defines a new class of tolerant mutant hereafter referred to as *tolI*. Likewise, mutant JK 114R3 defines a new class of resistant mutant referred to as *cir*.

Effect of colicin I on protein synthesis. Under conditions in which treatment of strain JK 114 with colicin Ia causes an inhibition of incorporation of [^{14}C]leucine into protein, protein synthesis in JK 114R1 is not inhibited (Fig. 3). These results are similar to those obtained when the effect of colicin on cell growth is determined by measuring increases in cell turbidity. Again, colicin Ia has no effect on strain JK 114R1. Similar results are seen with mutant JK 114R3.

Physiological properties of mutants. Since the physiological properties of JK 114R3 did not differ from the parental strain, its properties will not be discussed further. Although JK 114R1 grew with the same mean doubling time in TB broth as the parental strain, stationary phase occurred at a lower density (Fig. 4). This result is quite reproducible and was also found in cultures growing in synthetic medium containing glucose as the primary carbon source. A determination of growth yields, measured as turbidities, of the strains growing aerobically on media containing limiting concentrations of glucose showed (Fig. 5A) that the aerobic growth yield of strain JK 114R1 was lower than that of the parental strain. A similar experiment, in which succinate was substituted for glucose in the growth medium, shows that the

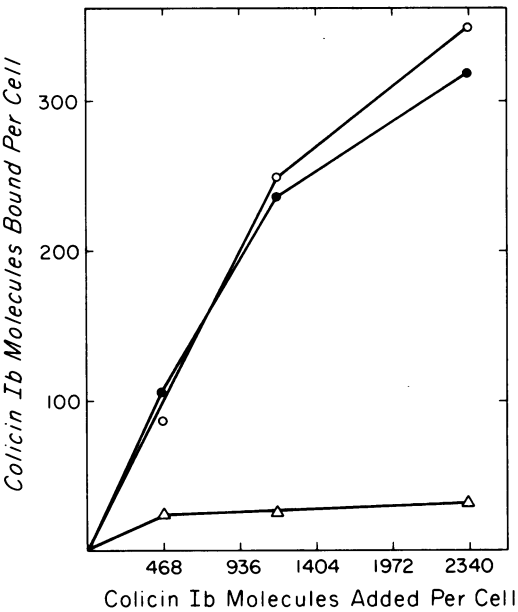

FIG. 2. *Adsorption of [^{125}I]colicin Ib to parental strain and colicin insensitive mutants JK 114R1 and JK 114R3. Various amounts of [^{125}I]Ib (5.3 × 10⁶ counts per min per nmol, 7 mol of iodine per mol of Ib, 0.83 μg/ml) were incubated with 3 × 10⁸ to 4 × 10⁸ cells of each strain for 30 min at 37 C. The amount of colicin Ib adsorbed was determined by the filter paper assay described previously (17). At the highest level of adsorption (100-μliter input) survivors were 2 and 50% for strains JK 114 (O) and JK 114R1 (●), respectively. Strain JK 114R3 (Δ) showed 100% survivors.*

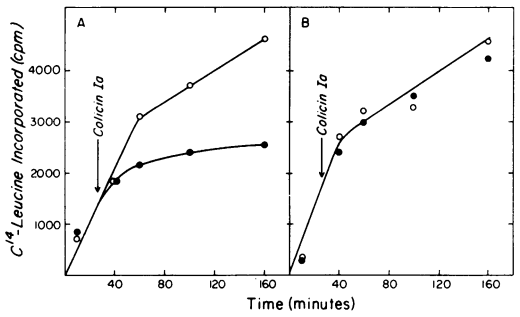

FIG. 3. *Effect of colicin Ia on protein synthesis in parental strain JK 114 and colicin I insensitive mutant JK 114R1. [^{14}C]leucine (57.6 mCi/mmol; final concentration, 1.15 μg/ml) was added to a log-phase culture of each strain (cell concentration approximately 1.5 × 10^8/ml) growing in TB broth at 37 C. Colicin Ia (final concentration, 0.1 μg/ml) was added 25 min later. At various times, samples were diluted into an equal volume of 10% trichloroacetic acid, heated for 15 min at 90 C, and chilled; the radioactivity incorporated was then measured by filtration onto glass fiber filter paper. (A) Strain JK 114: control, O; colicin treated, ●. (B) Strain JK 114R1: control, O; colicin treated, ●.*

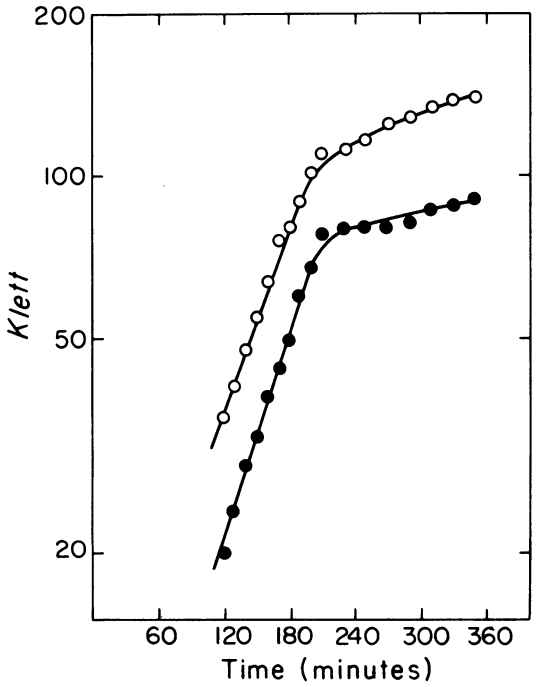

FIG. 4. *Growth curve of strains JK 114R1 in tryptone broth. Overnight cultures of each strain were diluted 1:50 into fresh tryptone broth and incubation carried out at 37 C with shaking. At various times the turbidity was determined in a Klett-Summerson colorimeter (filter 42). JK 114, O; JK 114R1 ●.*

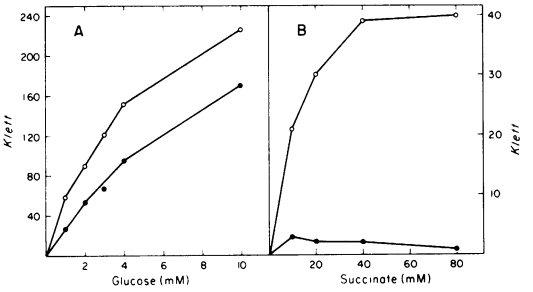

FIG. 5. *Growth yield of strains JK 114 and JK 114R1 at various glucose or succinate concentrations (A). A 0.005-ml volume of an overnight culture of the desired strain grown in M63 medium containing 0.15% glucose and the appropriate growth requirements was added to 5 ml of fresh medium containing the indicated amounts of glucose. After 22 h of incubation at 37 C with shaking, the turbidity of the culture was determined as in Fig. 4. A further 4 h of incubation showed no increase in Klett over that observed at 22 h. JK 114, O; JK 114R1, ●. (B) The method is the same as that described in A except that succinate replaced glucose as the carbon source.*

colicin I tolerant mutant cannot utilize succinate as a carbon source (Fig. 5B). Other experiments show that, unlike the parental strain, the mutant cannot utilize acetate. Preliminary experiments, in which glucose or succinate-dependent oxygen consumption was measured in whole cells, have shown that the respiratory rates in strains JK 114 and JK 114R1 are similar. This demonstrates that JK 114R1 retains a functional electron transport chain. This leads to the intriguing possibility that JK 114R1 cannot generate energy oxidatively.

Sensitivity of tolI mutant to azide and PEA. In many respects the mode of action of colicin I is similar to that of azide. Both inhibit macromolecular synthesis and a variety of active transport systems, while at the same time causing an increase in the accumulation of α-methyl glucoside (Gilchrist and Konisky, manuscript in preparation). It was, therefore, of interest to determine the sensitivity of the colicin I tolerant mutant to azide. Various amounts of sodium azide were added to growing broth cultures of JK 114 and JK 114R3, and the subsequent growth increment was measured after 290 min of incubation (Fig. 6). Whereas 1.25 × 10^{-3} M sodium azide completely inhibited growth of JK 114, strain JK 114R1 grew to a level corresponding to 43% that was attained by the same strain incubated in the absence of azide. Mutational analysis has shown that azide and phenethylalcohol (PEA) resistance are closely related (29). Strain JK 114R1 is less

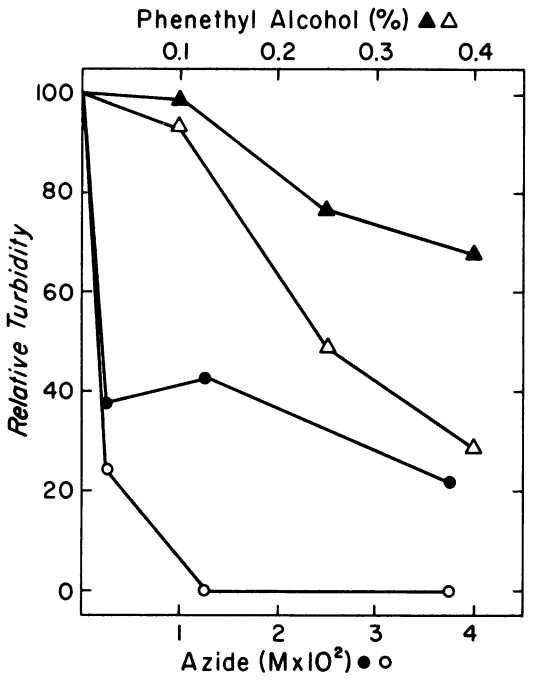

FIG. 6. *Effect of azide and phenethylalcohol on growth of strains JK 114 and JK 114R1. The appropriate strain was grown in tryptone broth at 37 C with shaking to a Klett of 15. Various amounts of azide were then added and incubation continued for an additional 290 min. At this time the Klett increment of each culture was determined. For each azide concentration, the datum is expressed as the Klett increment relative to the control culture containing no azide. The identical method was used in the case of phenethylalcohol. JK 114; azide, ○; PEA, △. JK 114R1: azide, ●; PEA, ▲.*

sensitive to PEA than its parental strain (Fig. 6). It is possible that these dissimilar agents have identical or interrelated targets.

Although the one *tolI* mutant which we have isolated exhibits azide resistance, the reciprocal relationships do not hold. For example, we have isolated a mutant of strain JK 114 which exhibits the same degree of azide resistance as *tolI*, but is not colicin I tolerant. Furthermore, strains JK 138 and JK 139 which are isogenic, except that JK 138 is azide resistant, are equally sensitive to colicin I.

Preliminary mapping of cir and tolI loci. The map positions of the *cir* and *tolI* loci were determined by mating each mutant with HfrKL16. In the mating to JK 114R3, *purC*$^+$ *str-r* recombinants were isolated and tested for the presence of the unselected markers *his*$^+$, *trp*$^+$, and *cir*$^+$. The position of the *cir* locus was determined by a semi-log plot of the frequency of unselected distal markers among *purC*$^+$ *str-r* recombinants versus the known map position of the marker in question (Fig. 7). Based on this method, the map position of the *cir* locus is approximately 39 to 41 min on the *E. coli* genetic map (27).

The position of the *tolI* locus was determined by a similar method (Fig. 8). In this case,

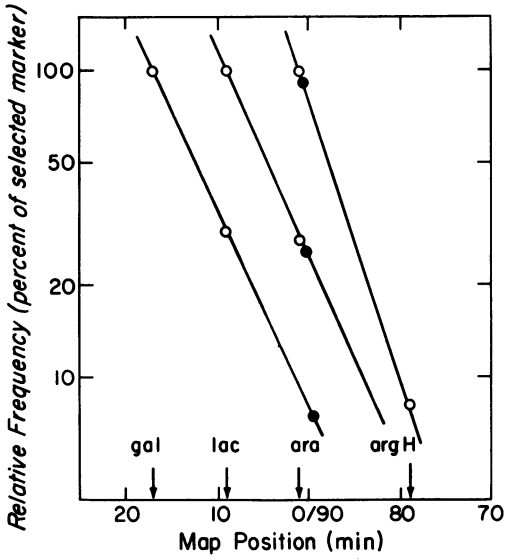

FIG. 7. *Mapping of cir locus. The mating was carried out as described in Materials and Methods. The location of the closed circle on the gradient line gives the approximate map position of cir. Sixty recombinants of each class were studied.*

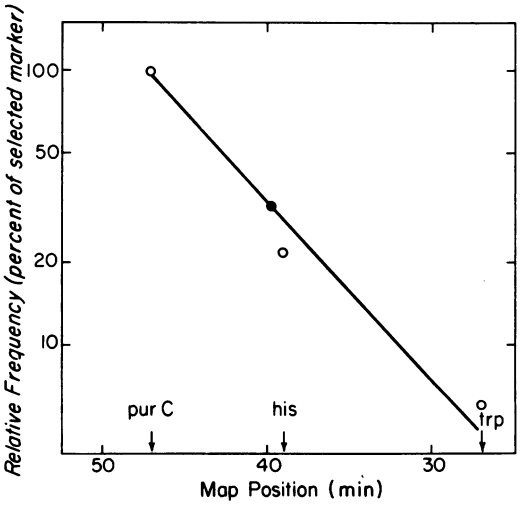

FIG. 8. *Mapping of the tolI locus. The procedure is as described in Fig. 8. 272 purC*$^+$ *str*^{r} *recombinants were examined.*

recombinants selected for *gal*$^+$ *str-r*, *lac*$^+$ *str-r*, or *ara*$^+$ *str-r* were checked for co-transfer of an unselected distal marker, as well as colicin sensitivity. When the data from all three determinations are aligned on the graph, one can see that in all three cases the *tolI* locus extrapolates to a map position of approximately 90 min.

DISCUSSION

The isolation and characterization of colicin tolerant mutants has the potential of being a powerful tool in furthering our understanding of colicin action. One would hope that the identification of structural and/or metabolic alterations in such mutants would serve to identify functions necessary for colicin action, and, thus, to shed light on the mechanism of action itself.

We report the isolation of a tolerant mutant selected for its insensitivity to colicin Ib which we propose to name *tolI*. Although the *tolI* mutant is greater than 10-fold less sensitive to colicin Ib than the parental strain, it retains full capacity to adsorb colicin Ib or the closely related colicin Ia at colicin concentrations lethal to the parental strain. Furthermore, the association constant for the interaction of [^{125}I]colicin Ib and its receptors is indistinguishable in the two strains.

The *tolI* class can be distinguished from all *tol* classes so far reported by several criteria. No other class tested is tolerant to colicin Ib. We have found that *tolI* is specifically tolerant to colicin Ib and the closely related colicin Ia. This is not surprising since both share a common mode of action (19), adsorb to common receptors (16), and are structurally related (15). On the other hand, *tolI* was found to be fully sensitive to colicins E1, E2, E3, K, and B as well as to colicins A, C, and bc246. Genetic analysis has localized the *tolI* locus to 89-1 min on the *E. coli* genetic map. This position is distinct from the position of all previously described *tol* mutations, except *cet* (89 min) which confers specific tolerance to colicin E2 (28). The *tolI* mutant does not seem to exhibit the surface changes exhibited by other *tol* and *cet* mutants. *tolI* is no more or less sensitive to sodium dodecyl sulfate, deoxycholate, or ethylenediaminetetraacetic acid than its colicin I sensitive parent.

One would predict the isolation of tolerant mutants with either altered "biochemical targets" or alterations in the colicin "killing pathway." Such mutations would be expected to confer on the strain tolerance to all colicins of the same mode of action. For example, one could envisage a change in the bacterial membrane, conferring tolerance to those colicins proposed to act on the membrane. The *tolC* mutation may be of the latter type. Colicins A, bc246, E1, K, Ia, and Ib can be grouped in a similar mode of action class based on their similar effects on macromolecular synthesis and transport functions (9, 10, 11, 19, 21, 24). However, the isolation of tolerant mutants singly insensitive to colicin E1 (*tolC*), bc246 (*tolG*), K (*tolI*), and Ia and Ib (*tolI*) clearly demonstrate that colicins which superficially show the same mode of action do, in fact, differ in the underlying basis for the observed modes of action. It should be noted, however, that just because such mutants show normal binding does not rule out the possibility of a receptor-based change which could lead to a tolerant phenotype. For example, transmission of the "killing signal" from the receptor to a secondary target could be blocked by mutation. However, it should be noted that receptor mutations for colicins E1, K, and I do not map near the corresponding *tol* mutation (27). Furthermore, it is difficult to imagine how an alteration in the colicin I receptor, which is a component of the cell wall (17), could lead to the physiological properties seen in the *tolI* mutant.

The physiological basis for colicin I tolerance is unclear. *tolI* shows reduced growth yields on glucose and is unable to utilize succinate or acetate. In this regard, the mutant could be defective in any one of a wide variety of functions necessary for utilization of these substrates as a sole source of carbon. These results suggest that some aerobic pathway must be operative for colicin I sensitivity. The phenotype of *tolI* is in some respects similar to that found in certain *E. coli* K-12 mutants which are unable to couple electron transport to oxidative phosphorylation. Such mutants, termed *unc*, exhibit low aerobic growth yields when grown on glucose and are unable to utilize succinate or malate (4, 5). However, both the *uncA* and *uncB* mutations have been localized to about min 73.5 on the *E. coli* K-12 chromosome and are, thus, genetically distinct from *tolI*. Furthermore, *uncA* is not colicin Ia tolerant (Konisky, unpublished experiment).

Guterman has described the isolation of mutants which had concomitantly lost sensitivity to colicins B, I, and V (13). Such strains, named *exb*, were shown to excrete the iron chelator enterochelin, which presumably interacts with these colicins, preventing adsorption (14). We report here the isolation of another class of mutants unable to adsorb either colicin Ia or Ib. The genetic locus for such colicin resistance is

approximately 41 min on the *E. coli* K-12 genetic map. We propose to name this locus *cir*. *Cir* retains full sensitivity to colicin B, and is genetically well separated from the two classes of *exb* mutants, *exbA* and *exbB*, which map at 25 and 58 min, respectively. We suggest that the *cir* locus is a structural gene for the colicin I receptor.

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