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Pleiotropic Properties and Genetic Organization of the *tolA,B* Locus of *Escherichia coli* K-12

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Colicin-tolerant mutants of *Escherichia coli* K-12, which map near *gal* at 17 min (*tolA,B* mutants), have been isolated and characterized. These mutants exhibited a very broad spectrum of phenotypic changes consistent with the interpretation that they are cell surface mutants. In addition to being colicin-tolerant and sensitive to deoxycholate and ethylenediaminetetraacetic acid, *tolA,B* mutants are sensitive to vancomycin, bacitracin, and dodecyl sulfate. The *tolA,B* mutants from most strains also formed mucoid colonies at 30 C on nutrient agar plates and had a greatly increased plating efficiency for lysis-defective *S* mutants of bacteriophage λ. Complementation analysis showed that the four phenotypic groups of *tol* mutants that map near *gal* fall into three complementation groups: *tolP*, *tolA*, and *tolB*. Recombination analysis by three-factor crosses established the order of the three groups as *tolP-tolA-tolB —gal*. Because of the wide variety of phenotypic changes that accompanies mutation to colicin tolerance, revertants were isolated to test whether single or multiple mutations were involved. The reversion analysis, as well as other genetic criteria, confirmed that only single mutations were involved, suggesting that these pleiotropic changes are a consequence of a single change in the *E. coli* cell surface.

The bacterial cell surface is a complex structure, consisting of a heterogenous mixture of proteins, lipids, and carbohydrates. An understanding of the function of each of these different molecular species will require the isolation and characterization of a wide variety of mutants with altered cell surfaces.

This genetic analysis has already revealed one common feature of these mutants that may be significant for an understanding of structure-function relationships in the cell surface: a large proportion of cell surface mutants, in both microbial and mammalian cells, exhibit a pleiotropic phenotype (9, 13, 14, 16, 18, 22, 23, 34; J. A. Wright, *personal communication*). These mutants show physiological alterations not only for the selected marker but also for other properties that seemingly were not involved in the selection procedure. This could be due either to interactions that occur between different molecules in the cell surface, or to multiple mutations. The possibility that multiple mutations have occurred, each giving rise to a single phenotypic change, must be considered for two reasons. First, mutagens are commonly used in mutant isolation, and there is good evidence that the commonly used mutagen, *N*-methyl-*N'*-nitrosoguanidine, induces multiple unselected mutations in *Escherichia coli* (10, 13). Second, the effects of cell surface genetic changes on cellular physiology, and possible interactions between various cell surface mutants, are both poorly understood, so that selection procedures might unwittingly be selecting for multiple mutations. Unfortunately, in very few instances have the necessary genetic studies been carried out to settle this question. Where genetic analysis has been done, the results frequently indicate that more than one mutation is responsible for the observed phenotype (13, 18).

This study was undertaken to examine in more detail the question of pleiotropy and to establish further physiological properties that might be altered in cell surface mutants. To do this, we have investigated the genetic organi-

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zation of a particular group of cell surface mutants, colicin-tolerant mutants of *E. coli* K-12.

Colicins are protein antibiotics that kill sensitive cells by first adsorbing to specific receptor sites on the cell surface. This adsorption is then rapidly followed by specific, intracellular changes leading to cell death (24). Studies on their mode of action suggest that colicins remain at these extracellular adsorption sites and act from there (20, 25). To explain how a single extracellular colicin molecule can bring about rapid intracellular changes, a general model of colicin action has been proposed (19, 24). According to this model, colicins act indirectly by triggering changes in the cytoplasmic membrane, which then acts as a "transmittor" for the colicin.

While this view of colicin action has been complicated by recent experiments with colicin E3 (5, 6), the model has been useful in predicting the existence of a class of cell surface mutants that are insensitive to the killing action of one or more colicins because of a genetic change in some aspect of this transmission system. These mutants, called colicin-tolerant mutants, can be distinguished from colicin-resistant mutants, which have lost or altered their colicin receptor sites; colicin-tolerant mutants still possess these adsorption sites, and thus are tolerant for some other reason. Mutation in several genes on the *E. coli* chromosome can give rise to colicin tolerance (7, 12, 16, 22, 26, 27, 31, 34); these mutants differ from each other in their genetic position, their pattern of colicin tolerance, and their physiological properties. All of the colicin-tolerant mutants that have been examined show physiological alterations indicative of cell surface changes (7, 16, 22, 34; B. Rolfe et al., Bacteriol. Proc., p. 50, 1971).

One particular group of *tol* mutants, linked to *gal*, are either completely or partially tolerant to one or more colicins. We shall refer to this group of genes as the *tolA,B* locus, as was done by Taylor (32). The *tolA,B* mutants have been shown (22) to be sensitive to the cationic detergent deoxycholate (DOC) and the chelating agent ethylenediaminetetraacetic acid (EDTA). In this paper, we describe further pleiotropic properties of these mutants, and initial studies on the genetic organization of the *tolA,B* locus.

MATERIALS AND METHODS

Media. For mating experiments, P1 transduction, and testing for sensitivity to the male-specific phage MS-2, L broth, and agar were used (2). Otherwise, Difco nutrient broth (NB) and agar (NA) were used (11). Eosin-methylene blue (EMB)-galactose plates contained (per liter): agar, 10 g; tryptone, 10 g; yeast extract, 1 g; KH_2PO_4, 2 g; NaCl, 5 g; galactose, 10 g; eosin Y, 400 mg; and methylene blue, 65 mg. EMB-O plates were identical to EMB-galactose plates except that no galactose was added. For genetic crosses and the construction of certain strains, minimal agar (MA) plates were used. MA contained (per liter): agar, 15 g; KH_2PO_4, 6.8 g; $(NH_4)_2SO_4 \cdot 7H_2O$, 1.0 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01 g; $FeSO_4$, 0.0005 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and thiamine hydrochloride, 5 mg. The *p*H was adjusted to 7.0 with KOH. Required amino acids were added to a concentration of 100 μg/ml. Deoxycholate-nutrient agar (DOC-NA) plates were nutrient agar plates containing 0.375% DOC (w/v).

Strains. The bacterial and phage strains used in this study are listed in Table 1.

Preparation of colicins. Colicinogenic bacteria from an overnight culture were diluted 1:100 into L broth and incubated at 37 C until they reached a cell density of about 2×10^8 cells per ml. Growth was monitored by measuring the optical density (OD) of the culture in a Coleman Junior spectrophotometer at 650 nm. An OD of 0.12 corresponds to a cell density of about 2×10^8 cells per ml. Mitomycin C was then added to a final concentration of 0.5 μg/ml. The culture was incubated for a further 4 hr and then centrifuged; the supernatant fluid was frozen at −25 C in 2-ml portions. The colicin preparations obtained in this way were then titered either by a spot test, or by a killing curve (28). Titers of 10^{11} to 10^{12} killing particles/ml were usually obtained. One killing unit was defined as the amount of colicin that kills one bacterium.

Isolation of colicin-tolerant mutants. No mutagen was used in the isolation procedures. A 0.1-ml amount of an overnight culture was spread over the surface of NA plates that had previously been spread with either colicin E3 or colicins E3 and K. The isolation plates were incubated for 36 hr at 37 C. Surviving colonies were then tested for their sensitivity to the bacteriophages BF23 and T6. Clones that were sensitive to both of these phages were purified several times and tested further.

Mating procedure. Overnight cultures of both donor (Hfr or F′) and recipient F^- strains were diluted 1:20 in fresh L broth and were grown to an OD of 0.12 at 37 C. The two cultures were then mixed in a volume ratio of one donor to nine recipients, and the mating mixture was incubated at 37 C with gentle swirling for 2 hr. When necessary, the cells were then washed several times in phosphate-buffered saline (PBS; 11) and diluted in PBS before plating.

Construction of recA strains. The *recA* derivatives of the AB1133 *tol* mutants used in this study were prepared by mating Hfr KL16-99 (*recA1*) with AB1133 F^- *tol*$^-$ *his*$^-$ strains and selecting for *his*$^+$ streptomycin-resistant recombinants on MA plates. Approximately 50 of these *his*$^+$ recombinants were transferred to two NA plates, one of which was irradiated with ultraviolet (UV) light. After incubation at 37 C, the recombinants that were UV-sensitive (usually about 5 of the 50) were picked off the unir-

TABLE 1. *Bacterial and phage strains employed*[a]

Strain	Relevant properties[b]	Immediate source
E. coli		
W602	F⁻, *gal*⁻, *bio*, *thr*, *leu*, *str*ʳ	C. Fuerst
HfrH121	Hfr, *gltA*, *gal*⁺, *str*ˢ	B. Sanwal
KL16-99	Hfr, Bl, *recAl*, *str*ˢ	D. Hoar
QD5003	SuIII⁺, permissive host for λsusS₇	S. McClure
CR63	Permissive host for T4 amber mutants	R. Josslin
DH61 (F_1' gal)	F′, *tol*⁺ donor, *pro*, *met*, *recAl*, *str*ˢ	D. Hart
AB1133	F⁻, *gal*⁻, *his*, *pro*, *arg*, *thr*, *leu*, *str*ʳ	M. Nomura
JC411 (col E1)	Produces colicin E1-K30	W. A. Cramer
W3110 (col E2)	Produces colicin E2-P9	D. Helinski
W3110 (col E3)	Produces colicins E3-CA35 and I	D. Helinski
K23K (col K)	Produces colicin K	S. E. Luria
O6:H16,23 (col A)	Produces colicin A	S. E. Luria
Bacteriophage		
MS-2	Male-specific phage	D. Hoar
λ⁺, λc, λv, λ*CI*857		R. W. Reader
λ*CI*857susS₇, λsus*R*5	Lysis-defective λ mutants	R. W. Reader
BF23	Same receptor as E-group colicins	P. Reeves
T4		R. Josslin
T4am*t*A3, T4am*t*B5	Lysis-defective T4 mutants	R. Josslin
T6	Same receptor as colicin K	C. Fuerst

[a] Amino acid and vitamin requirements: *arg*, arginine; *glt*, glutamate; *leu*, leucine; *met*, methionine; *pro*, proline; *thr*, threonine; *his*, histidine; B_1, vitamin B_1; *bio*, biotin.

[b] The symbol *gal*⁻ or *gal*⁺, inability or ability to use galactose as a carbon source; *str*ʳ, chromosomal resistance to streptomycin; *recA1*, mutation preventing formation of normal recombinants.

radiated master plate, purified, and then retested for the presence of the *tol* mutation and the *recA* mutation.

Phage techniques. Sensitivity to BF23 and T6 was tested by cross-streaking the bacterial strain against the phage on NA plates, and incubating overnight at 37 C. Sensitivity to MS-2 was determined in a similar manner except that L agar plates (15) or EMB-galactose plates were used. L agar plates were best scored after 4 to 6 hr, whereas EMB-galactose plates still gave clear results after overnight incubation.

Complementation. F′ *gal* episomes carrying various *tolA,B* mutations were constructed as follows. The episome from DC61 (F′ *gal*) was transferred into W602 *tol* mutants by selecting for *gal*⁺ streptomycin-resistant diploids on EMB-galactose-streptomycin plates. As has been previously reported (22, 26), *tol*⁺ is dominant to a *tolA,B* mutation, so that these diploids will be phenotypically *tol*⁺; that is, they will be colicin-sensitive and DOC-resistant. These diploids were then spread-plated onto EMB-galactose plates containing colicin E3. The *gal*⁺ (i.e., dark purple) survivors were then cross-streaked against phage BF23 to check for the presence of the adsorption site for colicin E3. BF23-sensitive clones were then tested for the presence of the sex factor by cross-streaking them against the male-specific phage MS-2 on L agar plates. MS-2-sensitive colonies were purified on EMB-galactose plates and used in complementation tests. As will be shown in the Results, the episomal *tolA,B* mutations obtained in this manner are genetically identical to the original *tol* mutation on the chromosome.

Complementation tests were performed by mating the presumed homodiploid of W602, isolated as described above, with *tolA,B* mutants isolated in AB1133. To determine quickly whether two mutations complemented each other, the *tol*⁺ phenotype was used as the selected marker, by plating the mating mixture directly on DOC-NA plates. Selection of stable *gal*⁺ diploids was also done, with *tol* as the unselected marker, by plating 0.1 ml of the 10^{-4} dilution of the mating mixture onto EMB-galactose plates.

Isolation of revertants. DOC-resistant revertants were isolated by spreading 10^8 cells onto DOC-NA plates. Resistant clones were then purified two times on DOC-NA plates before further testing. Vancomycin-resistant revertants were obtained by diluting log-phase cells into an equal volume of prewarmed NB containing vancomycin at 100 μg/ml. The culture was allowed to grow for 18 hr, and then appropriate dilutions were spread onto NA plates. Individual clones were purified two times on NA plates before further testing. Sodium dodecyl sulfate (SDS)-resistant revertants were isolated in a similar manner with SDS at a final concentration of 0.5% (w/v). All revertants were tested for, and had, the original auxotrophic markers of their parent, AB1133.

Chemicals. Mitomycin C was obtained from the Kyowa Hakko Kogyo Co., Tokyo, Japan. Vancomycin was purchased as Vancocin (vancomycin hy-

drochloride) from Eli Lilly & Co., Toronto, Canada. Bacitracin was obtained from Mann Research Laboratories, New York, N.Y. Antibiotics discs were from Difco. SDS was obtained from BDH Chemicals, Poole, England. Sodium deoxycholate was from Fisher Scientific Co.

RESULTS

Isolation of tolA,B mutants. Mutants isolated on the basis of tolerance to colicin E3 or K, or to both, also acquire complete or partial tolerance to several other colicins not used in the selection procedure. These mutants can be classified into four phenotypic groups, according to their pattern of colicin tolerance (22, 26, and see Table 2). The mutants used in this study are listed in Table 3 according to the parent from which they were isolated and their mutant and complementation groups. Experiments to be described below showed that these mutants fall into three complementation groups. Following the suggestions of Demerec et al. (8), we propose to call these cistrons *tolP* (P for partially tolerant), *tolA*, and *tolB*.

Antibiotic and detergent sensitivity of the tolA,B mutants. Mutations affecting the cell surface might be expected to alter the mutant cell's response to a wide variety of surface-active agents. It has already been shown that *tolA,B* mutants are very sensitive to the detergent DOC and to the chelating agent EDTA (22). We further tested them and found that *tolA,B* mutants were also sensitive to the anionic detergent SDS and to the antibiotics vancomycin and bacitracin (Fig. 1).

The addition of 10^{-2} M $MgCl_2$ completely abolished the sensitivity of *tolA,B* mutants to vancomycin (Fig. 1b). In other systems, magnesium has been shown to reduce the ability of vancomycin to inhibit cell growth and peptidoglycan synthesis in vitro (3); magnesium also reduces the capacity of vancomycin to bind to isolated cell walls of *Bacillus subtilis* (4).

TABLE 2. *Patterns of colicin tolerance of four phenotypic groups of tolA,B mutants*[a]

Class	Colicins				
	K	E1	E2	E3	A
tol$^+$	s	s	s	s	s
tol IIa	pr	pr	pr	pr	pr
tol II	r	r	r	r	r
tol III	s	r	r	r	r
tol IIIa	s	pr	pr	pr	pr

[a] Symbols: s, sensitive; r, resistant; pr, partially resistant.

TABLE 3. *Classification of the tolA,B mutants of E. coli used*

Mutant group	Complementation group	Parent			
		W602	AB1133	Hfr H	Hfr B-11
IIa	P	413[a]	12, 305, 507	634	603
II	A	617	9	614, 619	609, 809
III	B	515	503	638	301
IIIa	B	916	1	810	

[a] Figures represent the *tol*$^-$ mutant numbers.

$ZnCl_2$ (5×10^{-5} M) was added to NB for all of the bacitracin growth curves because zinc has been shown to increase greatly the antibacterial potency of bacitracin (33). We have verified this observation for the *tolA,B* mutants. Although we found that *tolA,B* mutants are slightly more sensitive to zinc than *tol*$^+$ cells (*unpublished data*), the concentration of zinc used in these experiments (5×10^{-5} M) was low enough not to have any effect, by itself, on the *tolA,B* mutants.

Because the phenotype of other colicin-tolerant mutants can be reverted to wild type by the addition of 10^{-1} M Mg^{2+} ions (7), we examined the effect of both $MgCl_2$ (10^{-1} M) and $ZnCl_2$ (5×10^{-5} M) on the *tolA,B* mutants, as measured by their sensitivity to colicin E3. These experiments showed that these ions do not affect the colicin tolerance of the mutants.

The data shown in Fig. 1 were obtained with *tol* mutants isolated from AB1133. Similar results have been obtained with mutants from W602 and HfrH121.

Colony morphology. *E. coli* has a distinctive colony morphology regardless of the temperature or medium of growth. Some of the mutations that affect cell surface biosynthesis might be expected to lead to an alteration in this distinctive appearance. It has been shown that *cap*, or *lon*, mutants have a mucoid colony morphology when grown at 30 C or on a minimal medium (21). These mutants, which map at 11 min on the *E. coli* chromosome, have also been shown to have elevated levels of several of the enzymes involved in cell wall biosynthesis (21). The *tolA,B* mutants isolated from AB1133, HfrB11, and 594 were also very mucoid when grown on NA plates at 30 C or on minimal medium at 30 or 37 C (Fig. 2). The same mutants grown on LB plates, or on NA plates at 37 C, were identical to *tol*$^+$ cells in colony morphology.

Plating of lysis-defective phage mutants. The bacterial cell surface plays a critical role in the development of a bacterial virus. After

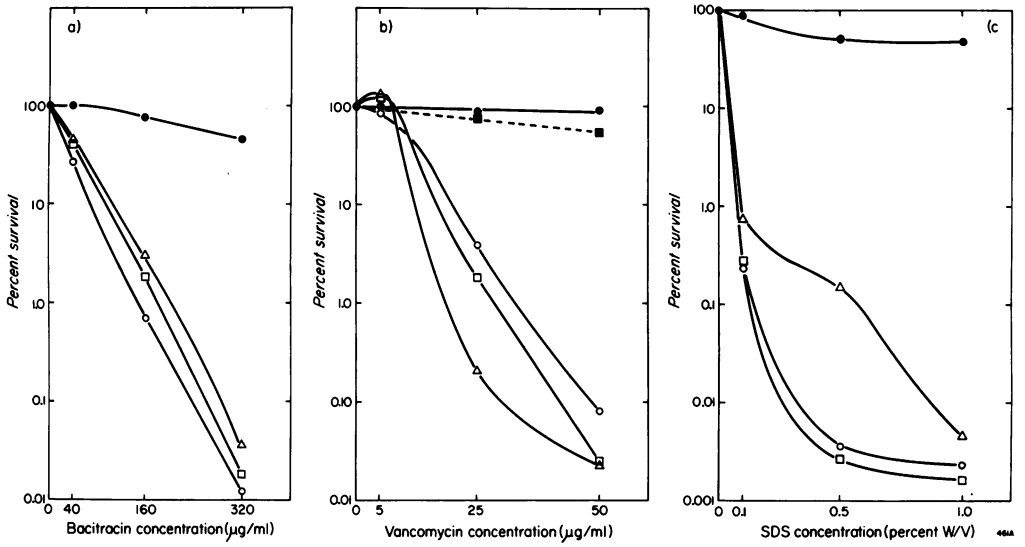

FIG. 1. *Effect of various surface-active agents on tolA,B mutants. A 1:20 dilution of an overnight broth culture of each strain was made into 2 ml of nutrient broth containing various concentrations of the agent. Cells were grown at 37 C for 4 hr, at which time viable counts were measured by spreading suitable dilutions on NA plates. Symbols:* ●, *AB1133 tol⁺;* ○, *tolP12;* □, *tolA9;* △, *tolB503;* ■, *(in b) tolA9 plus* 10^{-2} *M* $MgSO_4$.

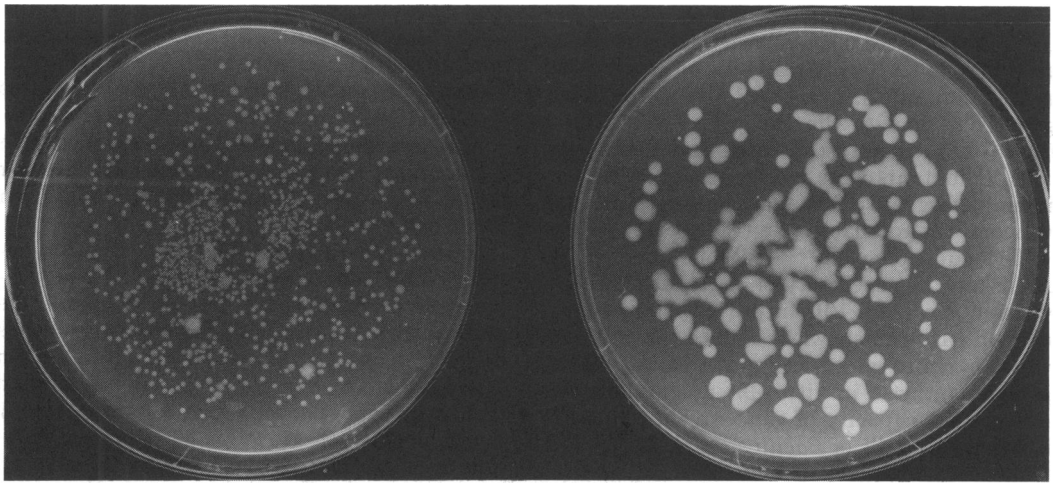

FIG. 2. *Colony morphology of AB1133 tol⁺ and AB1133 tolB503. An NB culture of each strain was diluted, and 0.1 ml was spread on two NA plates which were incubated overnight at 30 C. Left plate, AB1133; right plate, tolB503.*

the lytic cycle is completed, the infected cell usually lyses, releasing progeny phage. This lysis mechanism is not a passive event but, rather, requires the presence of at least two (for λ and T4) phage-coded proteins to destroy the integrity of the cell surface. One might expect, therefore, that in a cell that already had a cell surface altered by mutation, one or more of these phage degradative functions might not be required. We therefore tested the ability of two lysis-defective phage mutants of λ and T4 to plate on the *tolA,B* mutants (Table 4).

Phage mutants defective in endolysin, λ*R* and T4*e* mutants (11, 17), showed no increase in plating efficiency on *tolA,B* mutants. The same was true of T4*t* mutants. Only the λ*S* mutants plated 1,000-fold more efficiently on *tolA,B* mutants than on *tol⁺* cells. T4*t* mutants, which are analogous to λ*S* mutants in that both phage functions can be replaced by

TABLE 4. *Plating efficiency of lysis-defective phage mutants on tolA,B mutants*[a]

Bacterial strain	Phage strain				
	λv	λ*R*	λ*S*[b]	T4	T4am*t*A3
QD5003 ..	1.0	1.0	1.0	—[c]	—
CR63	—	—	—	1.0	1.0
AB1133 ..	0.85	1.0	3.9×10^{-5}	0.9	$<10^{-4}$
tol P12 ...	0.68	1.0	0.067	0.95	$<10^{-4}$
tol A9	0.64	1.0	0.073	0.87	$<10^{-4}$
tol B503 ..	0.63	1.0	0.064	0.91	$<10^{-4}$
tol B1	0.86	1.0	0.078	0.95	$<10^{-4}$

[a] Plating efficiency of λv, λsus*R*5, and λCI_{857}susS_7 were defined as 1.0 on QD5003. QD5003 is not a permissive host for λsus*R*, so that a plating efficiency of 1.0 actually is 10^{-4} times that on TC600.

[b] The plaque size of λCI_{857}susS_7 on the *tolA,B* mutants was much smaller than on QD5003.

[c] Test not performed.

chloroform (17, 29), showed no increase in plating.

The ability of the *tolA,B* mutants specifically to "suppress" the λ*S* mutation, and no others, suggests that this is not merely due to lysis of fragile cells and the subsequent release of progeny phage. If this were the case, one might expect *tolA,B* mutants to plate the other lysis-defective phage mutants also. This suggests that the λ*S* gene product might have a specific effect on the *tolA,B* gene products, which is particularly interesting, since *S* is thought to damage the cytoplasmic membrane (30).

Because of the wide variety of identical physiological changes that occur in these mutants, regardless of which phenotypic group the mutation belongs to, we were interested in examining more closely the genetic organization of the locus.

Linkage analysis. We have confirmed previous work that showed that the *tolA,B* locus is closely linked to *gal* (12, 22, 26). Conjugation experiments with Hfr × F⁻ crosses showed a 100% linkage between *gal* and *tolA, B* and vice-versa. P1 transduction experiments indicated about 50% linkage between any of the *tolA,B* mutants and *gal*, which agrees with the work of others (22, 26).

In the above experiments, colicin sensitivity or tolerance was used as the *tolA,B* marker. To test whether the physiological properties of *tolA,B* mutants were due to a single mutation, a P1 lysate grown on AB1133 *tol*⁺ was used to transduce various AB1133 *tolA,B* mutants to DOC resistance on DOC-NA plates. One hundred of these transductants were then tested for their sensitivity to colicin, their resistance to vancomycin, bacitracin, and SDS, their colony morphology, and their λ*S* plating ability. The results indicated that all of these properties were 100% linked to DOC resistance, and thus were probably due to a single mutation.

We have isolated mutants with deletions extending from *gal* to the *tolA,B* locus by using a double selection procedure described previously (27). All of these deletion mutants require nicotinic acid and are *aroG*. Conversely, we have tested several of the deletion mutants isolated by Adhya et al. (1) for markers in this region. Several of these mutants are *nadA*, *aroG*, but still *tol*⁺. Thus, the order of the loci must be *tolA,B-nadA-aroG-gal*, as shown in Fig. 3.

Fine structure map of the tolA,B locus. To establish the order of the *tol* mutants, a series of three-factor crosses was carried out between *gal*⁺ Hfr donors carrying various *tol* mutations and *gal*⁻ F⁻ recipients carrying different *tol* mutations. Selection was made directly for *tol*⁺ by plating the mating mixture on DOC-NA plates. A minimum of 150 of these DOC-resistant, *tol*⁺ recombinants were then transferred to EMB-galactose plates to determine whether they were *gal*⁺ or *gal*⁻. As shown in Fig. 4, a cross should result in a high incidence of *gal*⁺ recombinants if the Hfr parent carries a *tol* mutation that is farther from *gal* than the *tol* mutation in the F⁻ recipient. Conversely, if the Hfr *tol* mutation is closer to *gal* than the F⁻ mutation, a double crossover is necessary to give a *gal*⁺ recombinant. The results of these crosses are shown in Table 5.

From these three-factor crosses, the order of the four major phenotypic classes of *tol*

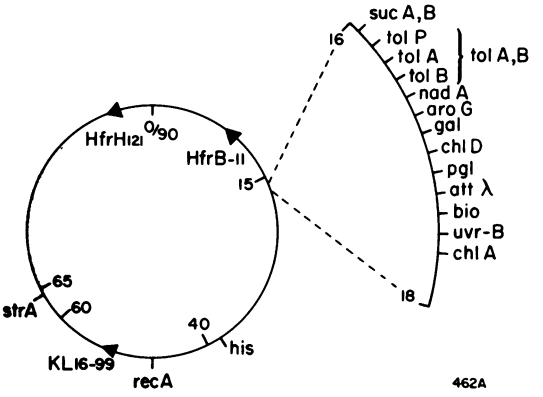

FIG. 3. *Genetic map of Escherichia coli, showing pertinent markers according to Taylor (32). Arrows indicate the origin and directions of transfer of the Hfr strains used.*

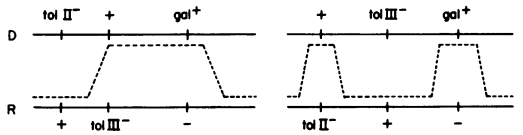

FIG. 4. *Rationale of three-factor crosses shown in Table 5. The broken lines represent the crossing-over necessary for the formation of (DOC^r, gal$^+$) recombinants. D, Hfr donor; R, F$^-$ recipient.*

TABLE 5. *Ordering of tol mutations by three-factor crosses*

tol mutation in		Percentage of *tol*$^+$ recombinants inheriting donor *gal*$^+$ marker	Order indicated
gal$^+$ donor	*gal*$^-$ recipient		
IIa-634	II-617	95.9	IIa-II
IIa-634	III-515	95.1	IIa-III
IIa-634	IIIa-916	95.0	IIa-IIIa
II-809	IIa-403	38.0	IIa-II
II-619	III-503	79.0	II-III
II-809	III-515	68.4	II-III
II-609	IIIa-1	81.6	II-IIIa
II-619	IIIa-916	79.9	II-IIIa
III-301	IIa-305	6.8	IIa-III
III-301	IIIa-1	78.3	III-IIIa
III-638	II-617	40	II-III
III-638	IIIa-916	34.9	IIIa-III

mutants is either IIa-II-III-IIIa or IIa-II-IIIa-III. The relative positions of III and IIIa have not been established, possibly because *tol* III and *tol* IIIa are in the same cistron (see below).

Complementation. F′ *gal* episomes carrying various *tolA,B* mutations (F′ *tol* episomes) were prepared as described in Materials and Methods, and were used to test for complementation in two ways.

In the first procedure, W602 (F′ *tol* mutants) were mated with either W602 or AB1133 *tolA,B* mutants, and *tol*$^+$ progeny were selected directly by plating the mating mixture on DOC-NA plates. DOC-resistant colonies can arise either through true complementation in *trans*, or through recombination between the episome and the chromosome. These two possibilities can easily be distinguished. First, a recombinational event leading to DOC resistance will be much rarer than complementation in *trans* between the episome and chromosome. Second, the possibility of recombination between the episome and chromosome can be drastically reduced by making the F$^-$ recipient *recA*, as described in Materials and Methods.

The results of such a series of crosses is shown in Table 6. As expected, three different types of crosses were obtained. First, a very high number of DOC-resistant progeny was seen (++ in Table 6), regardless of whether the female was *recA*$^+$ or *recA*. The second class of crosses (+ in Table 6) is the result of recombination between the episome and the chromosome, since the number of DOC-resistant progeny could be reduced to zero by making the recipient *recA*. In certain crosses, regardless of whether the F$^-$ recipient was *recA*$^+$ or *recA*, no DOC-resistant progeny were seen (− in Table 6). This was always the case when an F′ *tol* was crossed with the female originally used to construct the F′ *tol*. The fact that not even a recombinational event between the episome and chromosome mutations can give rise to a DOC-resistant colony suggests either that the two mutations are in identical nucleotide pairs or that one is a deletion overlapping the other. All of the episomal *tol* mutations were isolated as spontaneous events (as described in Materials and Methods). In addition, the F′ *tol* episomes can recombine with other mutants in the same cistron, suggesting that these episomal *tol* mutations are point mutants.

To investigate the behavior, in complementation tests, of some of the pleiotropic charac-

TABLE 6. *Complementation of tol mutations*[a]

Chromosome		Episome				
Group	Allele	Wild	IIa, 413[b]	II, 617	III, 515	IIIa, 916
IIa	413	++	−	++	++	++
	507	++	+	++	++	++
	507-*recA*	++	−	++	++	++
	12	++	−	++	++	++
	12-*recA*	++	−	++	++	++
	305	++	+	++	++	++
	305-*recA*	++	−	++	++	++
II	617	++	++	−	++	++
	9	++	++	−	++	++
	9-*recA*	++	++	−	++	++
III	515	++	++	++	−	+
	503	++	++	++	+	+
	503-*recA*	++	++	++	−	−
IIIa	916	++	++	++	+	−
	1	++	++	++	+	+
	1-*recA*	++	++	++	−	−

[a] Matings were performed as in Materials and Methods. Complementation was measured by plating suitable dilutions onto DOC-NA plates, and calculating the number of DOC-resistant colonies per milliliter, expressed as a percentage of the number of F′ donor cells per ml. Symbols: ++, 5 to 15%; +, 0.003 to 0.03%; −, less than 10^{-5}%, i.e., less than one DOC resistant colony per 10^7 male donor cells.

[b] Group and allele.

teristics of *tolA,B* mutants described above, stable partial diploids of the *gal-tolA,B* region were constructed by selecting for *gal*$^+$ progeny on EMB-galactose plates. Because no counterselective marker was available to eliminate the W602 (F′ *gal*) donors, it was necessary to distinguish among the four colony types that would be present after a mating: W602 (F′ *gal*) donors, AB1133 *recA* recipients, AB1133 (F′ *gal*) *recA* progeny, and W602 cells that had transferred their F′ to AB1133. In reconstruction experiments, it was observed that AB1133 (F′ *gal*) *recA* clones were easily distinguished from the other colony types present; W602 (F′ *gal*) cells are very dark purple, and AB1133 (F′ *gal*) cells lack the characteristic sheen of *gal*$^+$ cells. They can, however, still be distinguished from AB1133 itself (and W602), as they are larger, slightly darker, and more orange in color. Furthermore, only the AB1133 (F′ *gal*) *recA* progeny will be both MS-2- and UV-sensitive. Thus, potential heteroploids obtained in this manner were first checked for their MS-2 and UV sensitivity and then purified once on EMB-galactose plates. Stable heteroploids from each cross were then grown up in NB, and were tested for colicin, DOC, and vancomycin sensitivity, colony morphology at 30 C on NA plates, and efficiency of λ*S* plating (Table 7).

The results in Tables 6 and 7 indicate that the *tolA,B* mutants described in this paper

TABLE 7. *Complementation of tolA,B mutants*[a]

Chromosome		Episome				
Group	Allele	Wild	IIa, 413[b]	II, 617	III, 515	IIIa, 916
IIa	12-*recA*	++	−	++	++	++
	507-*recA*		−		++	++
	305-*recA*		−		++	
II	9-*recA*	++	++	−	++	++
III	503-*recA*	++		++	−	−
IIIa	1-*recA*	++	++	++	−	−

[a] Complementation was measured in stable partial diploids, constructed as described in the Results. Diploids were purified and scored for their phenotype as follows: −, no complementation; ++, strong complementation. No entry means the test was not performed. A ++ means that the diploid was sensitive to the colicins E1, E2, E3, K, and A, resistant to DOC and vancomycin, *tol*$^+$ in appearance on EMB-O plates (i.e., light pink), nonmucoid at 30 C on NA plates, and showed low plating efficiency of λ_{c1857} sus S_7. Vancomycin sensitivity was determined with Difco antibiotic sensitivity discs containing 30 μg of vancomycin.

[b] Group and allele.

TABLE 8. *Reversion analysis of tolA,B mutants*[a]

Strain	Colicins	DOC	SDS	VA	Morphology	λ*S* plating
tolP12	pr	s	s	s	M	+
Revertants						
P12 DOC^r,						
P12 SDS^r,						
P12 VA^r	s	r	r	r	NM	−
tolB503	r	s	s	s	M	+
Revertants						
B503 DOC^r,						
B503 SDS^r,						
B503 VA^r	s	r	r	r	NM	−

[a] Revertants were isolated as described in Materials and Methods. Abbreviations: M, mucoid; NM, nonmucoid; VA, vancomycin; for the others, see Table 2 and Materials and Methods.

fall into three complementation groups: *tol* IIa (*tolP*), *tol* II (*tolA*), and *tol* (III, IIIa) (*tolB*). Furthermore, Table 7 shows that the phenotypic properties of the mutants all behave as a single gene. That is, if two mutants complement each other for one property, then they invariably complement for all the other properties associated with the *tolA,B* mutants. Conversely, if two *tolA,B* mutants fail to complement for one property, then they also fail to complement for any property.

Table 7 also shows that *tol*$^+$ is dominant to *tol* in these diploids for all of the physiological changes described in this paper, as well as just for colicin tolerance (22, 26).

Analysis of revertants. The results of the complementation analysis, as well as the fact that mutations in all three cistrons lead to the same phenotype, suggest that the "*tolA,B* phenotype" is pleiotropic, that is, the result of single point mutations. To test this further, we isolated revertants resistant to DOC, SDS, or vancomycin, as described in Materials and Methods. Revertants were isolated from *tol* III-503 and *tol* IIa-12, and their phenotype was examined. In all cases, the entire *tolA,B* phenotype, as well as the selected marker, reverted to wild type (Table 8). This again provides strong evidence that the wide variety of physiological changes that accompanies mutation to colicin tolerance is due to a single mutation.

DISCUSSION

The colicin-tolerant mutants described in this report are all pleiotropic and have physiological properties indicative of an altered cell surface. The pleiotropy of these mutants is probably the result of a single mutation since

(i) no mutagen was used, thus minimizing the chance of multiple mutations, (ii) linkage analysis indicated that the entire "*tolA,B* phenotype" is very closely linked to *gal*, (iii) complementation analysis showed that the pleiotropic effects all behaved as a single gene, and (iv) reversion analysis confirmed this.

The pleiotropic changes accompanying mutation in the *tolA,B* region appear to be a frequent property of cell surface mutants. This is particularly so for other colicin-tolerant mutants that have been described; *tolC*, *tol* IVI, and *cetC* mutants all exhibit their own unique pattern of pleiotropic changes (16, 34; B. Rolfe et al., Bacteriol. Proc., p. 50, 1971). For example, all of these mutants are DOC-sensitive, but only the *tolA,B* mutants plate λ*S* mutants (*unpublished data*). The *tolC* mutants are resistent to vancomycin and bacitracin, but are sensitive to the antibiotics erythromycin and lincomycin (*unpublished data*). Pleiotropic effects have also been observed in other types of cell surface mutants, both in *E. coli* and in cultured mammalian cells. This pleiotropy might be a reflection of the close relationships between the various constituents of the cell surface. Hence, genetic changes in one molecule on the surface might have a marked effect on other neighboring molecules, leading to a pleiotropic phenotype.

Very little information is available concerning the genetic control of the cell surface, or the effect of mutation in cell surface genes on cellular physiology. The *tolA,B* locus apears to be an advantageous system for studying these questions. Mutation in any of the three *tolA,B* cistrons leads to an identical phenotype (aside from the pattern of colicin tolerance). Genetic analysis of these mutants indicates that these cistrons are probably contiguous. It will be of central interest, therefore, to determine whether the *tolA,B* locus is an operon, and what the role of its gene products is in the *E. coli* cell surface.

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