THE *tolC* LOCUS IN *ESCHERICHIA COLI* K12

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Received September 23, 1970

THIS investigation was undertaken with the object of determining the chromosomal location and fine structure of the *tolC* locus. The *tolC* locus seems a particularly interesting one to study, because the gene product appears to be involved in the structure and function of the cell membrane, an organelle about which genetic studies have yielded very little information to date.

The locus is one of several that are involved in the cell's response to colicins. A mutation at any of these loci can lead to the colicin-tolerant phenotype, evidenced by the fact that the cell loses its sensitivity to particular colicins, although it retains the ability to adsorb them to the same extent as a colicin-sensitive cell (HILL and HOLLAND 1967; NOMURA and WITTEN 1967; NAGEL DE ZWAIG and LURIA 1967). Mutations at the *tolC* locus differ from those at other *tol* loci, in that they make the cell tolerant to colicin E_1 only, while it responds with full sensitivity to colicins E_2, E_3, and K, which are related to colicin E_1 in their adsorption receptors or mode of action (REEVES 1965). *tolC* mutants also remain fully sensitive to phage BF23. This is significant because there exists another class of mutations causing colicin resistance for a different reason: these mutations, which affect the cell wall and not the membrane (BHATTACHARYYA *et al.* 1970), lead to loss of the cell's ability to adsorb colicins E_1, E_2, and E_3, as well as phage BF23 (FREDERICQ and BETZ-BAREAU 1952; JENKIN and ROWLEY 1955; REEVES 1966).

The main characteristic of colicin-tolerant mutants which makes them of interest to students of the cell membrane is that the mutants appear to be altered in some part of the membrane between the outer surface where the colicin molecules are adsorbed and the internal sites where the affected metabolic systems are located (NOMURA 1964; LURIA 1964). Recent evidence has improved the grounds for believing this. Colicins adsorb to the outside of the cell, as shown by their sensitivity, even after adsorption, to digestion by trypsin [ŠMARDA (1965) showed this for colicin E_1; MAEDA and NOMURA (1966) for E_2; NOMURA (1964) for E_3; and NOMURA and NAKAMURA (1962) for colicin K]. Colicins adsorb to the cell membrane and not to the cell wall (ŠMARDA and TAUBENECK 1968; BHATTACHARYYA *et al.* 1970). The processes affected by colicins are membrane-mediated processes (FIELDS and LURIA 1969a, 1969b). In addition, tolerant mutants including those of the *tolC* class display pleiotropic effects such as hypersensitivity to certain dyes, drugs, and detergents (CLOWES 1965; NAGEL DE ZWAIG and LURIA 1967), suggesting that they have an altered cell membrane.

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TABLE 1

*Bacterial strains**

Strain		Relevant genotype	Reference
Escherichia coli K12			
K10	HfrCav	*str*^{s}	GAREN and LEVINTHAL 1960
KL16	Hfr	*str*^{s}	LOW 1965
AB311	Hfr	*thr*$^-$ *leu*$^-$	TAYLOR and ADELBERG 1960
AB313	Hfr	*thr*$^-$ *leu*$^-$	TAYLOR and ADELBERG 1960
AT2699	F$^-$	*argG6 metC58 thyA2 his-1*	TAYLOR and TROTTER 1967
X36	F$^-$	*argH purC*$^-$ *his*$^-$ *pyrF trp*$^-$	WOLF, NEWMAN and GLASER 1968
AT2475		*serA6*	TAYLOR and TROTTER 1967
NP315 (formerly h8)		*fda*	BÖCK and NEIDHARDT 1966
CA38		(Col I, Col E_3)	FREDERICQ and DELCOUR 1953
Salmonella typhimurium LT-2		*cysD36*	CLOWES and HAYES 1968
RC903		(Col E_1)	.....
RC906		(Col E_2)	.....

* The nomenclature used conforms with the proposals of DEMEREC *et al.* (1966) except for the modifications used by CURTISS (1967). The genetic markers are listed in the order in which they appear on the TAYLOR (1970) map (see also Figure 2). Only those markers relevant to this study are included, and mutations conferring resistance to phages and inability to ferment carbohydrates are omitted. Mating types are indicated only for strains which were used in conjugation experiments.

Strains K10 and AB311 were supplied by S. SILVER, KL16 by B. LOW, AB313 by D. SCHLESSINGER, AT2699 and AT2475 by A. L. TAYLOR, X36 by B. WOLF, CA38 by L. WENDT, NP315 by F. C. NEIDHARDT, and RC903 and RC906 by R. CLOWES.

Amber mutations at the *tolC* locus have been reported (NAGEL DE ZWAIG and LURIA 1969), indicating that the primary gene product is a protein. This research was undertaken since a fine-structure genetic map of this protein might be informative and lead to a genetic basis for understanding the colicin-response system and its involvement in structure and function of the cell membrane.

MATERIALS AND METHODS

Bacterial strains: The strains used and their relevant characteristics are listed in Table 1. A clear-plaque mutant of phage P1, kindly provided by D. APIRION, was used for transduction. Phage BF23 was a gift from R. C. CLOWES.

Media: L broth (LURIA and BURROUS 1957) was used for growing bacterial cultures; saline (0.85% NaCl) was used for resuspending centrifuged cells, and as dilution fluid. Solid media included L agar slants (L broth with 2% Bacto-Agar (Difco)), L top agar (L broth with 0.7% agar), and L agar plates (L broth containing 1.5% agar) to which additions of streptomycin (100 μg/ml), $CaCl_2$ (2.5 mM), acriflavine HCl (25 μg/ml), or sodium deoxycholate (0.05%) were made as required. The supplemented L agar plates were called STR, Ca, AF, and DOC plates, respectively; for Ca plates the L agar was usually diluted to 1.1% during addition of $CaCl_2$, in order to provide an optimally slippery surface for the preparation of phage lysates.

Selective media consisted of minimal agar plates (1.5% agar with M9 salts—6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl per liter of deionized water—and 0.2% glucose added separately), supplemented as required with L-arginine, uracil, hypoxanthine, and thiamine at 10 μg/ml, thymidine at 15 μg/ml, L-histidine and L-methionine at 25 μg/ml, L-tryptophan at 50 μg/ml, and streptomycin at 100 μg/ml.

Growth and maintenance of bacterial stocks: Stocks were maintained on L agar slants at 4°C,

transferred from slant to slant every 2–3 months, and purified before each use in genetic experiments by streaking out on L plates or selective plates and incubating overnight at 37°C. A single-colony isolate was picked, streaked, and grown again, and a second single-colony isolate picked and grown on a fresh slant for storage. Overnight cultures were grown in L broth with aeration at 37°C, diluted into fresh L broth, and grown to concentrations of 2×10^7/ml for Hfr strains in conjugation, $2–5 \times 10^8$/ml for F^- strains in conjugation, and $1–2 \times 10^9$/ml for transduction.

Selection of tolC *mutants:* Colicin E_1-resistant mutants were selected on colicin E_1-containing plates made by growing 200 colonies of RC903 on L agar plates overnight at 37°C and sterilizing the plates with chloroform vapors. Each plate was overlaid with 3 ml of melted L top agar (maintained at 45°C in a water bath) seeded with 0.3 ml of an overnight culture of the desired *Escherichia coli* strain. The plates were incubated a second night at 37°C, and resistant colonies were picked up from the top agar, and grown overnight in a "grid" pattern on a fresh plate. The grid was then replica-plated (LEDERBERG and LEDERBERG 1952) onto AF or DOC plates to test the selected colonies for the *tolC* (AF^s DOC^s) phenotype, and onto other appropriate test media to check their genetic characteristics. The *tolC* mutants obtained (i.e., those that were resistant to colicin E_1 and sensitive to AF or DOC) were purified at least two successive times by streaking out on L agar plates and isolating single colonies, rechecked for all relevant characteristics, and stored on L agar slants at 4°C.

Independent *tolC* mutants, specifically for use in intragenic mapping, were isolated in strain AT2699 by overlaying each colicin E_1-containing plate with a culture grown from a separate colony of AT2699 previously picked from a DOC plate, and ultimately using only one *tolC* mutant from each colicin plate (except for the pairs EW1a & EW1b and EW3a & EW3b, which were picked from the same plate and could have arisen from single events). These mutants were tested further by plating at least 10^{10} cells on DOC plates, and except where noted only those producing no DOC^r colonies in populations that size were used for mapping.

Names of mutants: In most cases, the mutants used in this study were *tolC* derivatives of published strains listed in Table 1, and are designated by numbers. Mutants 50–99 and 150–199 were isolated from K10, 200–299 from AB311, and 300–399 from AB313. 101 and 102 were the only *tolC* mutants isolated from X36.

In the case of the mutants used for intragenic mapping, on the other hand, strains were constructed by the author, and these have been tagged with the initials EW. Crosses between EW strains may be understood to be between strains isogenic in all respects except for the markers being studied in the cross. The standard set of seven mutants used in this way were EW1a, EW1b, EW2, EW3b, EW4, EW65, and EW308. The first five contain stable, independent *tolC* mutations (except for EW1a and EW1b, which may carry the same mutation) isolated in AT2699 and processed further as described under RESULTS. The last two contain *tolC* mutations 65 (isolated in strain K10) and 308 (isolated in strain AB313), transferred into strain AT2699 as described in RESULTS.

Scoring of genetic markers: Mutants and recombinants were scored after single-colony isolation by gridding and replica-plating as described above, with the following two exceptions. When very large numbers of recombinants had to be examined, during intragenic mapping, a direct replica-plating method similar to that of CLOWES and HAYES (1968) was adopted for the purpose of determining the number of *tolC*+ recombinants that had inherited the *metC*+ marker. The selective DOC plates themselves were used as masters, and replicated onto selective media lacking methionine (as well as onto DOC plates as controls) to make a direct count of the number of *tolC*+ recombinants that were *metC*+.

Colicin resistance and sensitivity were scored as follows: strains producing the desired colicins (E1, E2, and E3) were stabbed from overnight cultures near the periphery of L agar plates, incubated overnight at 37°C, sterilized with chloroform vapors, overlaid with the strains to be tested, and incubated a second night. Sensitivity of the test strain to the colicin was evidenced by a large, clear halo around the stab of the producing strain. Sensitivity to colicin K was tested using a solution of the purified colicin provided by M. JESAITIS (JESAITIS 1967). A loopful of this solution was spotted onto an L agar plate on whose surface was spread about 10^7 cells of the bacterial strain to be tested. After overnight incubation at 37°C an area of clearing indicated in-

hibition of growth of sensitive bacteria. Sensitivity to phage BF23 was assayed by streaking a loopful of a dilute suspension of the phage across such a spread bacterial lawn before incubation; plaques became clearly visible if the cells were sensitive.

Conjugation: The procedure of JACOB and WOLLMAN (1961) was followed, with modifications described by CURTISS *et al.* (1968) after these were learned.

P1-transduction: Phage P1 lysates were prepared on Ca plates by the method of SWANSTROM and ADAMS (1951), harvested after 4–6 hr of incubation at 37°C, stored overnight over chloroform at room temperature, and used for transduction within 36 hr. Crosses were always performed with phage prepared at least two successive times on the desired donor strain, to eliminate carry-over of phage grown on previous strains. Before use, each lysate was diluted ten-fold into saline and subjected to a dose of ultraviolet light sufficient to kill 98% of the phage (APIRION and SCHLESSINGER 1967). Equal volumes of the irradiated phage and late log phase cells were incubated with $CaCl_2$ and $MgCl_2$ (both at 2 mM) for 20 to 30 min at 37°C, centrifuged to remove unadsorbed phage and broth, resuspended in a small volume of saline, and plated on selective media. Cells alone and phage alone were always plated as controls.

For intragenic crosses a step was added to this procedure: After exposure to phage and centrifugation, the cells were resuspended in fresh L broth and aerated at 37°C for an extra 2 hr to allow time for recombination and expression of the *tolC*+ phenotype (DOC resistance), then recentrifuged, suspended in saline and plated as above.

In crosses using as donor the temperature-sensitive strain NP315, which grows normally at 30°C but not at 43°C, all steps (growth of donor cells, preparation of the lysate, adsorption of phage to recipient cells, incubation of selective plates, and incubation of grids of transductants) were carried out at 30°C. To test whether transductants had inherited the donor's temperature sensitivity, duplicate L agar replicas of the grid were incubated at 30°C and at 43°C.

RESULTS

Characteristics of mutants: Several hundred spontaneous mutants resistant to colicin E_1 were collected from the six strains most frequently used in this investigation. When these were screened for sensitivity to AF or to DOC, 13 to 33% were found to be AF sensitive and DOC sensitive (AF^s, DOC^s), except with strain X36, which, for a reason not understood, gave rise to only two *tolC* mutants (Table 2). Those mutants which were not AF^s and DOC^s were not systematically tabulated, since it was primarily *tolC* mutants which were sought, but 11 representative strains were tested and found to fall into at least two classes: 10 resembled the cell-wall type of mutant described in the INTRODUCTION in being resistant to colicins E_1, E_2, E_3, and phage BF23, but sensitive to colicin K. One of these was mapped near *argE*, and therefore probably resembles those observed before (JENKIN and ROWLEY 1955; REEVES 1966). The eleventh appeared to be a member of the *tol VI* class described by NOMURA and WITTEN (1967), which

TABLE 2

Frequency of tolC *mutants among colicin E_1-resistant isolates*

Parental strain	Number tested	AF^s DOC^s mutants found	
		Number	Percent
AB311	399	86	22
AB313	399	50	13
K10	98	33	33
X36	383	2	0.5

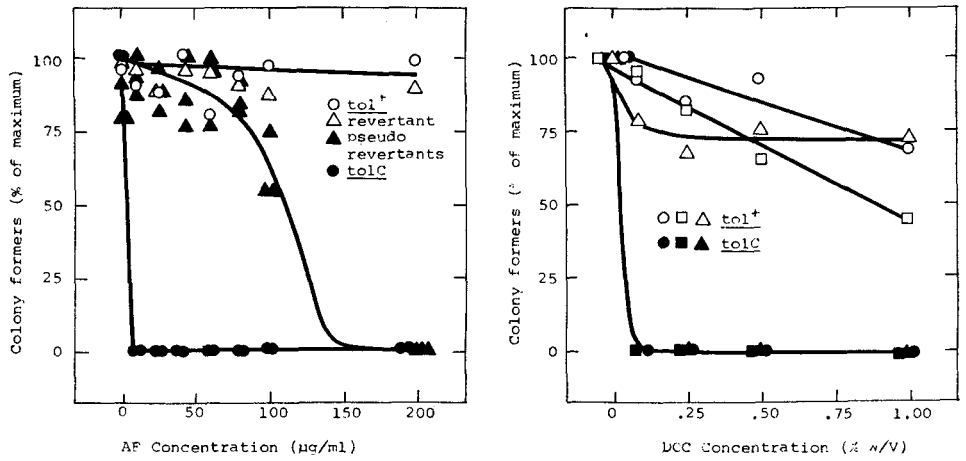


FIGURE 1.—Acriflavine and deoxycholate inhibition of mutants. Cells were plated on L agar containing graded concentrations of acriflavine (AF) and deoxycholate (DOC), incubated overnight at 37°C, and colonies counted. *AF inhibition*: Acriflavine at 5 µg/ml inhibits the two *tolC* mutants, and at 100–150 µg/ml distinguishes 3 "pseudorevertants" from the parent and "true" revertant strains which are still resistant at 200 µg/ml. The strains used were AB311 *tolC*+ and its *tolC* derivatives 203 and 204, a revertant of its *tolC* derivative 286, and "pseudorevertants" of 213, 223, and 238. *DOC inhibition*: DOC at 0.05% inhibits all *tolC* mutants, while *tolC*+ strains remain relatively resistant at 1.0%. The strains used were K10 (△), AB313 (○), and X36 (□), and their *tolC* derivatives 65 (▲), 308 (●), and 101 (■), respectively.

is resistant to colicins E_1 and E_2, but sensitive to colicin E_3 (and K) and phage BF23. The mutants selected and their characteristics are described in more detail in WHITNEY (1970).

The sensitivity of the *tolC* mutants to varying concentrations of AF and DOC in L agar plates was tested, and it was found that *tolC* mutant strains gave rise to less than 1% of the expected colonies in the presence of 5 µg/ml or more AF or 0.05% or more DOC, while *tolC*+ parents or revertants were resistant to at least 200 µg/ml AF and 1.0% DOC (Figure 1).

Approximate back-mutation rates for the *tolC* mutants were obtained by spreading 10^{10} cells from overnight cultures on AF or DOC plates. Colonies that appeared were picked, purified by streaking for single colonies, and retested for AF and DOC sensitivity; some were also picked into L broth, grown overnight, and tested for colicin sensitivity and resistance. Two cell types could be recovered from AF plates: some were "true" revertants, as judged by the fact that they had reverted in one step to AF and DOC resistance and to colicin E_1 sensitivity; and others were "pseudorevertants," which could grow into colonies on AF plates but not on DOC, and which, when tested, proved still resistant to colicin E_1. Their AF resistance was not as complete as that of true revertants: four representative pseudorevertants tested were sensitive to AF at concentrations between 100 and 200 µg/ml while the parental strains and true revertants were resistant at these concentrations (Figure 1).

The two classes of revertant (pseudo and true) arose with equal frequency

($\sim 10^{-8}$) among 134 *tolC* mutants studied, and were sometimes both observed to arise from the same *tolC* strain. 73 of the *tolC* mutant strains tested yielded no true (E_1^s) revertants on repeated tests; some of these probably carry deletions. Pseudorevertants were never seen to arise on DOC plates, and those that were picked from AF plates always proved DOC sensitive. In several cases *tolC* strains which yielded AF^r pseudorevertants at the typical frequency of $\sim 10^{-8}$ yielded no true revertants (on DOC) down to a frequency of 10^{-12}. These included deletion mutant 308, and represent partial physiological suppression of one of the pleiotropic effects of deletions in *tolC*. The genetic locus involved in the pseudoreversions has not been sought.

Mapping of the tolC *locus:* The relevant portion of the *Escherichia coli* genetic map is shown in Figure 2. The *tolC* locus first appeared on the map at a tentative position near the *his* locus (TAYLOR and TROTTER 1967), following preliminary mapping by CLOWES (1965) and by NAGEL DE ZWAIG and LURIA (1967); the latter investigators called it *tol VIII*. HILL and HOLLAND (1967), calling it *ref I*, mapped the same locus near *thyA*. My preliminary crosses confirmed the location of *tolC* between *strA* and *his*, and analysis of conjugational recombinants from a cross of K10 Hfr × X36 F^- showed *tolC* to be more closely linked to *purC* than to *his*. An interrupted mating between KL16 Hfr and X36 F^- showed *tolC* to be a late marker on the KL16 chromosome (WHITNEY 1970), and therefore between *strA* and *thyA*. The strain AT2699 F^-, marked at *argG*, *metC*, and *thyA*, was therefore utilized and crosses with a *tolC* mutant derivative of this strain and of AB313 Hfr showed *tolC* to be more closely linked to *metC* than to either *argG* or *thyA* (Table 3).

The linkage of *tolC* to *metC* was confirmed by transduction. $tolC^+$ strains carrying *argG* and *metC* (AT2699) and *serA* (AT2475) were used as donors; $tolC^+$ transductants were selected directly (on DOC plates) with four different *tolC* mutants as recipients, and in all four cases, methionine auxotrophy was inherited by about 5% of the transductants, while the *argG* and *serA* markers were not detectably cotransduced with *tolC* (Table 4). In other experiments an additional 14 *tolC* mutants were tested and showed cotransduction of *tolC* with *metC*. It remains to determine on which side of *metC* the *tolC* locus is situated. B. ROLFE (personal communication) has mapped *tolC* between *argG* and *metC* by analysis of recombinants from conjugation experiments, and his data support the position of *metC* at 58 minutes as in Figure 2. I had tentatively placed *tolC* between *metC*

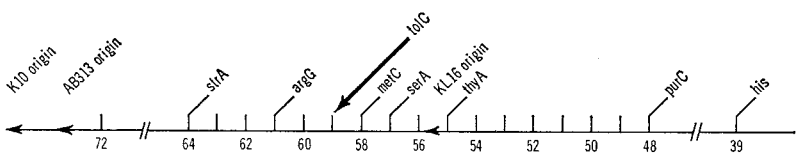


FIGURE 2.—The *his–str* region of the genetic map of *Escherichia coli* K12. The figure shows the positions of markers employed in this study; others are omitted. The design is revised from TAYLOR's (1970) genetic map of *Escherichia coli*, on which *tolC* was placed at 58, and *metC* at 59 minutes.

TABLE 3

Frequency of unselected markers in a cross of AB313 Hfr thr⁻ leu⁻ tolC × *AT2699 F⁻* argG metC thyA*

Selected markers	Number tested	Percent unselected markers *argG+*	*metC+*	*thyA+*	*tolC*
argG+	54	..	30	22	26
metC+	54	61	..	31	87
	187†	73	..	47	85
thyA+	37	51	65	..	65
argG+ metC+	108	..	..	33	91
metC+ thyA+	54	67	..	..	89

* AB313 was contraselected by omission of threonine and leucine from the plating medium.
† These were additional recombinants from a separate identical cross.

and *serA* by analysis of conjugation experiments involving loss of expected recombinants distal to *tolC* in conjugal transfer (WHITNEY 1970; TAYLOR 1970). However, as shown in Table 4, *serA* appears to be cotransducible with *metC* at a frequency of about 1% (4/318) and if this is the case, then *tolC* must lie on the opposite side of *metC* from *serA* as shown in Figure 2. [Note, however, that TAYLOR and TROTTER (1967) found no cotransduction (less than 0.3%) of *metC* with *serA*.] Therefore the order of these three genes is still not firmly determined.

The TAYLOR (1970) map tentatively locates another marker, *fda*, at 60 min in the space between *arg* and *metC*; if it does lie there, *fda* might be contransducible with *tolC*. The *fda* mutant NP315 was obtained from F. C. NEIDHARDT and an attempt made to cotransduce the two, using the mutant as donor and selecting DOC-resistant recombinants in strains EW65 and 308. No cotransduction was detected (0/208) but the genetic distances are sufficiently great that *fda* may be located near 60 min on the map and still not be cotransducible with *tolC* near 59 min.

Fine-structure map of the tolC *locus:* A set of seven *tolC* mutations was used to obtain the outlines of the fine-structure map. Five of these were isolated directly in AT2699; the other two (65 and 308) were crossed into AT2699 from 65 and 308 by selection and screening of *metC+* transductional derivatives. The five original AT2699 (*metC thyA*) mutants were transduced in two successive steps to *metC+* and *thyA+* for use as donors and were used directly as recipients. The other two were transduced to *thyA+* for use as donors and were used directly as recipients. Thus crosses between these strains (EW1a, EW1b, EW2, EW3b, EW4, EW65, and EW308) involved donors and recipients that were isogenic for all markers except those being studied.

Intragenic *tolC+* recombinants were selected directly on DOC plates; *thyA+* recombinants were selected on defined media lacking thymidine, and the ratio of *tolC+* to *thyA+* transductants was taken as a measure of the genetic distance between the two *tolC* mutations used in each cross. Where the donor and recipient were also *metC+* and *metC*, respectively (i.e., in all cases except where EW65 and EW308 were used as recipients), I measured the percentage of *tolC+* trans-

TABLE 4

Cotransduction of genetic markers in the tolC *region*

Marker carried by: Donor*	Recipient	Transductants inheriting unselected donor marker/ transductants tested		Cotransduction (percent)
metC	tolC65	3/53	15/291	5.1
metC	tolC68	6/108		
metC	tolC69	4/76		
metC	tolC202	2/54		
argG	tolC65	0/53	0/291	<0.3
argG	tolC68	0/108		
argG	tolC69	0/76		
argG	tolC202	0/54		
serA	tolC65	0/208	0/424	<0.2
serA	tolC68	0/33		
serA	tolC69	0/30		
serA	tolC202	0/47		
tolC65	serA	0/106		
metC	serA	4/318		1.3
argG	serA	0/318		<0.3

* Strains used were AT2475 for *serA* and AT2699 for *metC* and *argG*. Four different *tolC* mutants were used as indicated. When the recipient was *tolC*, *tol*+ transductants selected directly on DOC plates were scored for cotransduction of *metC*, *argG*, or *serA*. When the recipient was strain AT2475 *serA*, *serA*+ transductants were tested for cotransduction of *tolC*, *metC*, or *argG*.

ductants which had inherited the donor marker *metC*+ in reciprocal crosses, in order to determine the order of the two mutations with respect to *metC*. All possible reciprocal and self-crosses were performed at least twice each among these strains.

When the outlines of the map were clear, ten mutants from other strains (namely 69, 101, 102, 208, 223, 228, 303, 311, 314, and 330) were crossed, using them as donors only, against some or all of the original seven, without reciprocal or self-crosses as controls, but still normalizing *tolC*+ to *thyA*+ transductants. Not all possible combinations were tried, but all were crossed against EW2 and either EW1b or EW3b, and most were also crossed against EW308. Twelve, and probably all seventeen, of the mutations so far tested fall into four groups by the

simple criteria diagramed in Figure 3. One group, exemplified in the figure by EW1b, consists of stable mutations which do not recombine with each other or with EW3b, give very few recombinants with EW308, and many with EW2. A second set of stable mutations, of which EW3b is the best characterized, recombines with neither the first set nor EW308, and gives intermediate values with EW2. EW308 is unique so far, recombining with the first set but not with the second, and giving intermediate values with EW2. Finally, there is a set of mutations, similar to EW2, which recombine with all the others. Two of these (208 and 228) also revert to *tolC*$^+$ at a frequency of about 10^{-8} per cell. The members of each group are listed in the legend to Figure 3.

A more detailed map than that of Figure 3 can of course be drawn, and is shown in Figure 4. The genetic distances (expressed as *tolC*$^+$ transductants per 10^4 *thyA*$^+$ transductants obtained in the same cross) vary over five orders of magnitude, from 0.02 to about 150. Details of the way in which these numbers were averaged from identical crosses are given in the legend to Figure 4. Mutations were judged not to recombine when the value obtained was less than 0.09 in repeated crosses; whenever possible this value was pushed to 0.05 or lower. While this resolution was not ideal, since in two cases recombinants showed up at frequencies below 0.05, it was the best that could practically be obtained.

The mutations at the left end of the map are drawn as deletions, although only EW3b, EW4, and 101 are multisite mutations by the strictest criterion (i.e., that they give no recombinants with other mutations—EW308 and the group that includes EW1a, EW1b, EW65, and 102—that do recombine with each other), and their differing extents are tentatively determined by their recombination frequencies with EW308, on the assumption that these data, from a short genetic distance, are more accurate than those with the more distant EW2.

Five mutations are not shown in Figure 4; each has been used once in crosses but not pursued very far, since they appeared to fall into already defined classes. 69 and 223 resemble EW1b or EW3b: they yield *tolC*$^+$ recombinants per 10^4 *thyA*$^+$ recombinants at levels of 110 and 150 with EW2 but < 0.5 and < 0.3 with EW1b. 69 and 223 were not crossed against EW308. 303, 311, and 314 recombine with EW2 at levels of 17.9, 9.5, and 11.4, respectively, and at low levels with EW308—0.12, 0.07, and 0.01—and do not recombine with EW3b (< 0.05, < 0.04, < 0.02).

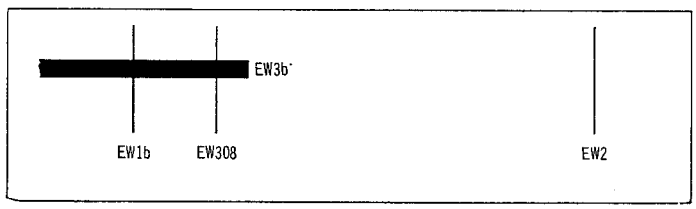


FIGURE 3.—Four classes of *tolC* mutation defined by intragenic recombination experiments. Similar to EW1b are EW65, EW1a, and 102, and probably 69, 223, 303, 311, and 314; the latter five were not fully characterized (see text). Similar to EW3b are EW4 and 101. Similar to EW2 are 208, 228, and 330. Both EW1b and EW3b are overlapping deletions, extending into the locus from the left.

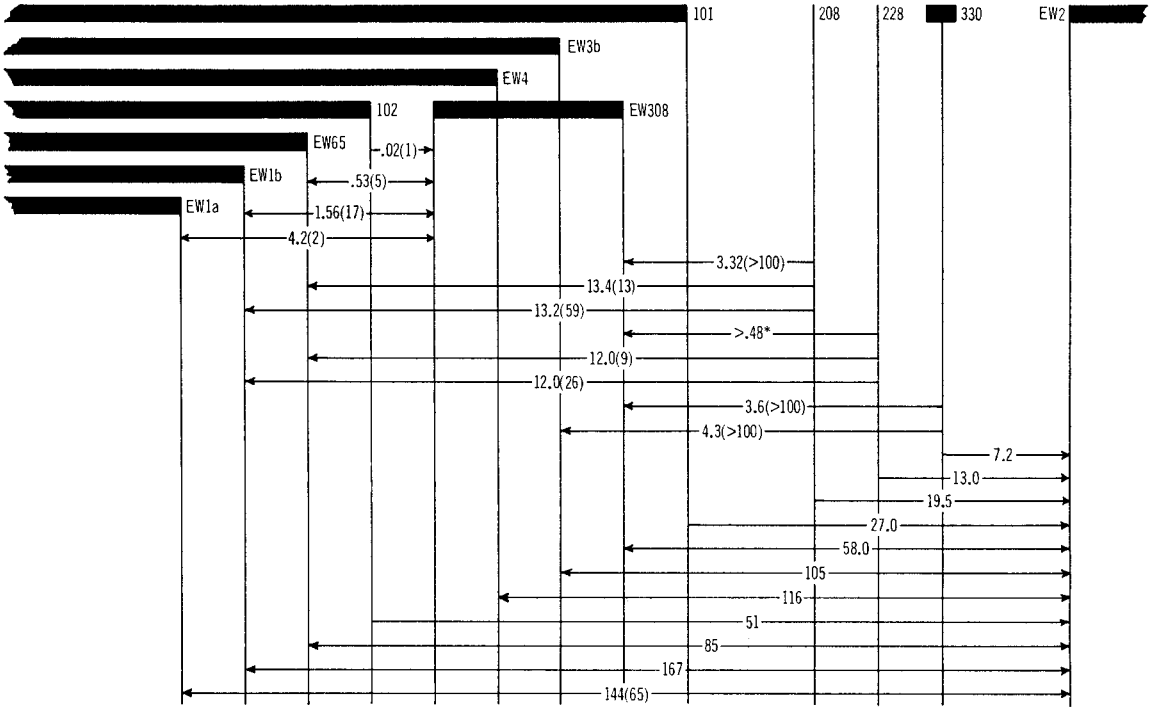


FIGURE 4.—Fine-structure map of the *tolC* locus. The map depicts the twelve best-studied mutations at the locus. Each value is given as *tolC*+ transductants per 10^4 *thy*+ transductants obtained from the same cross. Arrowheads point toward recipients in each cross; where reciprocal crosses were done, they point both ways. All values are averaged from two or, usually, more separate determinations, except those for 330, which was used only once, and that for the distance between 102 and EW308, which was estimated on the basis of only 1 *tol*+ recombinant. Averages were weighted in proportion to the number of *tol*+ recombinants obtained from each cross up to an arbitrary limit of 100, beyond which losses of accuracy in colony counts were considered to offset gains of statistical accuracy from the numbers of colonies counted. The number of *tol*+ recombinants from which each value was determined is given in parentheses except for most of those for distances to EW2, where *tol*+ recombinants numbered more than 100 in each case. *The value of > 0.48 for the distance from 208 to EW308 is a minimum for trivial reasons.

The three-factor data are weak, since *metC* is so loosely linked to *tolC*, but suggest that EW1b and the others like it are closest to *metC* and EW2 is furthest from *metC*. The most informative data were not obtained from reciprocal crosses, but from crosses of donors 208, 228, and 330 with EW3b on the one hand and EW2 on the other. In these crosses, large numbers of *tolC*+ recombinants were obtained and analyzed (both by conventional replica-plating of at least fifty from each cross, and by the direct method described under MATERIALS AND METHODS). Almost a twofold greater cotransduction of *metC*+ with *tolC*+ was found with EW3b as recipient than with EW2 (Table 5). Since the mutations 208, 228, and 330 lie between the mutations EW3b and EW2, two crossover events are required to give rise to *tolC*+ *metC*+ recombinants with EW3b and four crossover events

with EW2, if the order is as indicated in Table 5 and *metC* is to the left of the map in Figure 4. Reciprocal crosses between EW1b and EW2 gave approximately equal numbers of *metC*+ recombinants. EW308 never yielded more than 8 *tolC*+ recombinants in any one cross with mutations to its left in Figure 4, and could not be used as a *metC* recipient (since it was obtained by selecting *metC*+ transductants in AT2699). When EW308 was used as donor with recipient EW2, a value similar to those for the other three donors listed in Table 5 was obtained. This information, too, is consistent with a position for *metC* to the left of the locus as drawn in Figure 4.

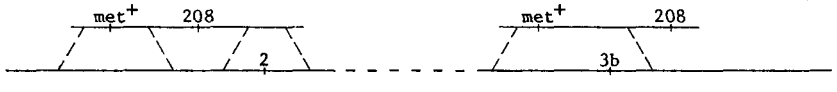
DISCUSSION

Suppression of tolC *mutations:* Amber-suppressible *tolC* mutants are known (NAGEL DE ZWAIG and LURIA 1969) but the pseudorevertants found in this study which are partially resistant to acriflavine must have another explanation. Several explanations are possible. One involves interactions between different proteins in the cell membrane. There are several reasons why this might be likely. First, deletion mutants (e.g., EW1b and EW308) give rise to pseudorevertants and it would be hard to imagine suppression of deletion mutations at the level of transcription or translation. Furthermore, only one of the pleiotropic effects of the *tolC* mutation is suppressed in the pseudorevertants, i.e., acriflavine sensitivity. The pseudorevertants remain fully sensitive to deoxycholate and resistant to

TABLE 5

Three-factor data from metC+ tolC × metC tolC *crosses**

	metC+ transductants found among total tolC+ transductants tested			
	Recipient EW3b		Recipient EW2	
Donor:	(Number)	(%)	(Number)	(%)
208	19/187	10	64/681	9
228	19/156	12	36/530	7
330	38/239	16	36/546	7
EW308	--	--	9/188	5



* Donors were *metC*+, recipients *metC*, *tolC*+ (DOC^r) recombinants were collected and analyzed as described in the text. No recombinants were obtained in crosses between EW308 and EW3b.

colicin E_1. And no deoxycholate-resistant pseudorevertants have been found in extensive tests. Interactions among the products of different genes controlling parts of the ribosome structure have been found (APIRION and SCHLESSINGER 1969), and this type of suppression may be common with complex structures such as the ribosome and the membrane.

Mapping of the tolC *locus: tolC* is cotransducible with *metC* and appears to be located at 59 min on the map as shown in Figure 2. In the conjugation data collected from crosses involving *tolC*, however, an anomaly was observed, which should be mentioned. If an Hfr *tolC* is crossed with an F^- *tolC*$^+$ and Hfr markers other than *tolC* are directly selected, the *tolC* allele appears in recombinants at frequencies lower than would be predicted. When an Hfr *tolC*$^+$ is crossed with an F^- *tolC*, and other Hfr markers are selected, the *tolC*$^+$ allele appears at higher frequencies than predicted. This effect was observed in every case where such comparisons were possible, in crosses involving two different Hfr and three different F^- strains (WHITNEY 1970). It may reflect a general tendency to lose recombinants carrying *tolC* mutations (semilethality).

Fine-structure map of the tolC *locus:* The detailed map of the *tolC* locus that can be constructed from these data (Figure 4) shows certain features. Many of the stable mutations used fall together at one end. Some of these can be distinguished from others since some (like EW1b) do, and some (like EW3b) do not, recombine with EW308; this is probably the first of many distinctions which will define each stable mutation uniquely.

EW1b and the other mutations in its class, and EW3b and the other mutations in its class, are considered to overlap for two reasons. First, they repeatedly gave no *tolC*$^+$ recombinants in experiments where 0.09 *tolC*$^+$ per 10^4 *thyA*$^+$ transductants or fewer would have been detected. In many cases the resolution was pushed to 0.02 per 10^4 *thyA*$^+$ transductants. Secondly, the distances measured from them to near mutations with which they do recombine show differences consistent with their terminating at different points.

The four mutations furthest left in Figure 4 could be ordered by the distances measured from their termini to EW308 or to EW2; both sets of numbers indicate that EW1a and EW1b terminate first, then EW65, then 102. Similarly, recombination values with the three central mutations agree that EW1b and EW65 terminate farther from the center than do EW308 and EW3b. The three central mutations in turn have not been assigned a definite order, but the order shown is consistent with the distances measured from them both to EW308 on the left, and to EW2 on the right. Finally, the distances measured between EW2 and the mutations closer to it (EW4 through 330) are in rough agreement with the order shown; there are discrepancies among the greatest distances but perhaps this is not unreasonable.

There remains a possibility that the nonrecombining mutations resembling EW1b and those resembling EW3b, or some of them, might recombine at a still lower frequency, since in two cases (described above), such events were observed. However, the eight mutations drawn as deletions at the left end of Figure 4 not only arose from independent events and in five different strains, but also yielded

no revertants in total populations of more than 10^{11} cells of each strain tested and therefore are undoubtedly deletions.

It also seems justifiable to draw EW308 as a deletion, since it does not revert at 10^{-12} frequency, and better additivity of the distances to its left and right is obtained this way than if it is considered a point mutation. When the appropriate point mutations are crossed against these deletions, they will probably sort out into a more rigorously defined array, with one or another point mutation to the left and/or right of each terminus.

The credit for the idea underlying this research, and for helpful guidance during its course goes to SIMON SILVER. TEREK SCHWARZ kindly suggested the use of deoxycholate, which made possible the fine-structure genetic study. My research was made possible by the consistent and reliable help of Mrs. MARY FRANCES MITCHELL. The isolation and preliminary mapping of many of the mutants used was done by NANCY WALLS. The author is also indebted to GERALDINE KNUCKLES and DAN CLARK for their generous help. The work was supported by Grants AI08062 from the U.S. Public Health Service and GB 5922 from the National Science Foundation. I was supported as a predoctoral trainee by an NDEA Title IV fellowship. Due to lack of funds, no reprints of this paper will be available.

SUMMARY

Spontaneous mutants resistant to colicin E_1 were isolated, and those sensitive to 25 μg/ml acriflavine and 0.05% sodium deoxycholate (*tolC* mutants) were studied. Back mutation of about half of the *tolC* mutants to colicin E_1 sensitivity and resistance to acriflavine and deoxycholate was observed; among the remainder and some of the reverting mutants, pseudorevertants were also observed, having partial acriflavine resistance but no detectable alteration in deoxycholate sensitivity or colicin E_1 resistance. Both revertants and pseudorevertants arose at a frequency of $\sim 10^{-8}$ in most cases. Among strains in which pseudorevertants to acriflavine resistance were observed there were several deletion mutants.—The *tolC* locus was mapped by conjugation at a position near *metC*. P1-transduction experiments showed the order of markers in the *argG–serA* region of the *Escherichia coli* genetic map to be *argG–tolC–metC–serA* from the following cotransduction frequencies: *argG–tolC* < 0.3%, *tolC–metC* 5.1%, *metC–serA* 1.3%, *tolC–serA* < 0.2%.—An intragenic map was constructed from reciprocal crosses by phage P1-mediated transduction, using seven independent nonrevertible *tolC* mutants. Ten additional mutants, including two point mutants, were crossed as donors with representatives of the first seven. The number of *tolC*$^+$ recombinants obtained in each cross was normalized to the number of *thyA*$^+$ transductants obtained from the same cross; genetic distances so obtained varied from 2×10^{-6} to 0.2 of the frequency of *thyA*$^+$ transductants, depending on which mutations were crossed. The map that resulted from these measurements and the assortment of the linked outside marker, *metC*, contained: a series of 12 deletions of varying extent entering the locus from the *metC* end; an additional deletion terminating at both ends within the locus, and overlapping three of the 12; and 4 other mutations, including the 2 point mutations, toward the other end of the map.

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