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Triornithine-resistant Strains of *Escherichia coli*

ISOLATION, DEFINITION, AND GENETIC STUDIES*

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SUMMARY

Triornithine-resistant (TOR) mutants were isolated from several strains of *Escherichia coli* K-12. All of the mutants were found to be oligopeptide transport-deficient (Opp⁻). The dipeptide transport system (or systems) was not altered. The rate of growth of these mutants, the morphology of the cells and their colonies and the sensitivity to the phages T_1, T_4, P1, and ϕ80 were similar to the parental strains, indicating no dramatic changes at the cell envelope. All Opp⁻ mutants tested showed 98% linkage to *trp* in sexual recombination, and 80 to 95% P1-mediated co-transduction frequencies. The *opp* gene is therefore the closest marker to the *trp* operon. A mutant with a deletion in the region *att* ϕ80, *tonB*, *trp* still contained the *opp* gene (or genes), indicating its appearance on the *cysB* side of the *trp* operon.

Tri-L-ornithine is a synthetic basic tripeptide which when accumulated by *Escherichia coli* specifically blocks protein biosynthesis, causing inhibition of growth (1–4). Previous reports have mentioned the isolation of spontaneous triornithine-resistant mutants in several strains of *E. coli* W, *E. coli* B, *E. coli* K-12, and *Salmonella typhimurium* (2, 5–10). Resistance to the action of the toxic tripeptide could arise for any of the following reasons: (*a*) a loss of the ability to transport the tripeptide, (*b*) increased intracellular peptidase activity, (*c*) derivatization of the toxic peptide, (*d*) change in sensitivity of the target site. However, in all cases examined to date, the resistance is attained through the loss of the ability to transport triornithine (5, 7, 8). This loss is accompanied by the inability to transport all peptides containing more than two amino acids (5–7, 9, 10). This finding, coupled with the observation that different oligopeptides compete for entrance into the cell, has led to the proposal that there is only one oligopeptide transport system in *E. coli* (6, 7). The observation that all the TOR[1] mutants that have been studied are found to be altered in their oligopeptide transport provides a facile means to select for oligopeptide transport-deficient mutants (Opp⁻). These Opp⁻ mutants might represent a family of strains having defects in different genes that are essential for the normal activity of the system. Genetic analysis of different Opp⁻ strains can be used, therefore, to study the complexity of the oligopeptide transport system.

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[1] The abbreviations used are: TOR, triornithine-resistant; Lys-*p*-F-Phe, L-lysyl-L-*p*-fluorophenylalanine; di-Lys-*p*-F-Phe, L-lysyl-L-lysyl-L-*p*-fluorophenylalanine; triornithine, tri-L-ornithine tetrahydrochloride; Pro-Phe, L-prolyl-L-phenylalanine; Pro-Phe-Lys, L-prolyl-L-phenylalanyl-L-lysine; *p*-F-Phe, *p*-fluorophenylalanine.

EXPERIMENTAL PROCEDURES

Chemicals—The peptides L-lysyl-L-*p*-fluorophenylalanine, and L-lysyl-L-lysyl-L-*p*-fluorophenylalanine were synthesized by T. E. Fickel of this department. The peptides were examined for purity by paper electrophoresis (2) and the amino acid content was confirmed by amino acid analysis. Tri-L-ornithine tetrahydrochloride, L-prolyl-L-phenylalanine, and L-prolyl-L-phenylalanyl-L-lysine were purchased from Miles-Yeda (Rehovot, Israel).

Bacterial Strains—Most of the strains used in this study were reisolated from one colony and tested for their nutritional requirements and their phage and drug resistance. The reisolated substrains were marked by different Roman numbers which were added to the name of the strain. All the triornithine-resistant mutants are spontaneous mutants that were isolated from triornithine-treated cultures according to methods described under "Results." They were all designated as TOR. The number after TOR refers to the individual isolates.

E. coli W (ATCC 9637), *E. coli* B (Weizmann Institute of Science, Rehovot, Israel), and *E. coli* Bb are wild types.

E. coli K-12 Hfr-H (Hfr-H) *thi-1*, *rel-1*, PO 1 (11) was a gift of Dr. Worcel from this department. Hfr-H-II-TOR-1, Hfr-H-III-TOR-1,2, and Hfr-H-IV-TOR-1,2 are spontaneous TOR mutants of this strain.

E. coli K-12 Hfr-3000 (Hfr-3000) *thi-1*, *rel-1*, λ⁻, PO 1 (11). This strain and its derivatives were used as the male in all our mating experiments. Hfr-3000-TOR-1,2,3,4 are spontaneous TOR mutants of this strain.

E. coli K-12 AB1157-*trp*^{del}-V (TD-V) is a substrain of a deletion mutant which was obtained by Dr. E. C. Cox of this University from *E. coli* K-12 AB1157. *E. coli* K-12 AB1157 is F⁻, *thr-1*, *leu-6*, *proA2*, *his-4*, *argE3*, *lacY1*, *galK2*, *ara-14*, *xyl-5*, *mtl-1*, *thi-1*, *supE44*, *sup-37*, *str-31*, *tsx-33*, λ⁻ (11). The deletion was obtained by selection with ϕ80 vir and colicin (12), and it includes the region from *att* ϕ80 to *trp*. This strain is therefore also *trp*⁻, ϕ80^r, col E^r, and T_1^r. The substrain was tested for

its deletion by its resistance to the phages of ϕ80 vir and T_1 (see "Results") and by its tryptophan requirement. TD-V-TOR-1, 2,3,4,5 are spontaneous TOR mutants of this strain.

E. coli K-12 KLF23/KL 181 (CGSC 4256) is a gift of Dr. B. Bachmann from Yale University. It is a male F′ strain. The episome F′123 contains the following genes: *rac*, *pyrF*, *cysB*, and *trp*. The chromosomal markers are: *pyrD34*, *thi-1*, *his-68*, *trp-45 recA1*, *mtl-2*, *xyl-7*, *malA1*, *galK35*, *str-113*, λ^r, λ^-.

Media—The minimal media used were Medium A (13), M-9 (14), and VB (15). The latter was used as a minimal medium (VB min) or with the addition of all the amino acids required by strain TD-V (threonine, leucine, proline, tryptophan, histidine, arginine) at a concentration of 50 μg per ml (VB full). Sometimes streptomycin (50 μg per ml) was added to the medium which was then called VB full Sm. The rich media used in these studies were L-broth (L-b) (16) and Medium H (16). Agar (1.5%) was added to media when preparing agar plates, whereas 0.7% agar was used in the top agar preparations.

Bacterial Growth—The rate of growth and the yield of bacteria were followed by measuring the change in optical density as a function of time in a Klett-Summerson colorimeter using a 660-nm filter. One hundred Klett units correspond to about 10^9 bacteria per ml. The bacteria were grown in Klett tubes containing 5 ml of medium, and were incubated with shaking (150 rpm) at 37°. The peptides were added to the sterile medium aseptically. VB full medium was used when measuring the toxicity of peptides whereas VB full *minus* proline (*Minus* Pro) was used when testing the nutritional effect of Pro-Phe and Pro-Phe-Lys.

Phage Sensitivity—The sensitivity of the different strains to phages was measured qualitatively by lysis of cells on agar plates. Bacteria from growing cultures were spread on H agar plates. After the plates were dry, a drop of concentrated phage suspension (around 10^9 per ml) was added. The lysis was determined after overnight incubation at 37°.

Mating—Exponentially growing cultures (L-broth with shaking at 37° to 2 to 4 $\times$ 10^8 per ml) of donors, Hfr-3000 and Hfr-3000-TORs, and recipient, TD-V, were mixed in a ratio of 1 Hfr:5 to 8 F^-. This was achieved by mixing 2.5 ml of fresh L-broth medium prewarmed to 37° with 0.5 ml of recipient culture to which 0.1 ml of the donor culture was added. The mixing was carried out in a 50-ml Erlenmeyer flask. The mating mixture was incubated without shaking at 37° for 2 hours. When scoring for recombinants, remating on the plates was avoided by using streptomycin (50 μg per ml) to select against Hfr cells.

Replica Plating—Master plates were prepared by picking colonies and placing them on a plate divided into squares. The grid contained 68 colonies per plate. The replications were carried out with sterile velvet pads.

P1 Transduction—Lysates of P1 were prepared according to the method of J. H. Miller (17). Transduction experiments were carried out by infecting 0.9 ml of exponentially growing cells (2 to 5 $\times$ 10^8 per ml) in L-broth + $CaCl_2$ (5×10^{-3} M), with 0.1 ml of P1 suspensions. Adsorption of phages was performed by incubation at 37° for 20 min. Sodium citrate, pH 7, 0.1 M (5 ml), was added to stop further infection. Cells were centrifuged and resuspended in 1 ml of sodium citrate. Samples (0.1 ml) were mixed with 4 ml of melted (45°) VB min top agar and immediately plated onto appropriate plates. Control experiments revealed the absence of donor survivors in the phage lysates and revertants in the recipient preinfected culture.

RESULTS

Isolation of Spontaneous TOR Mutants—Spontaneous TOR mutants have been isolated from several *E. coli* K-12, *E. coli* W, and *E. coli* B strains. Isolation has been carried out using two general procedures: (*a*) isolation from cultures of cells that had been grown after a long lag in minimal liquid medium in the presence of 50 to 100 μg of triornithine per ml and (*b*) direct selection of TOR mutants on minimal agar plates containing 100 μg of triornithine per ml.

As shown in Table I randomly picked isolated colonies from a culture of cells that had been grown in the presence of triornithine are almost entirely TOR mutants. Similarly, TOR mutants are obtained when plating 2 $\times$ 10^6 cells of *E. coli* W and *E. coli* B on minimal agar plates containing 100 μg of triornithine per ml. *E. coli* K-12-TD-V, however, sometimes shows a background of pinhead colonies when growing on VB full Sm plates containing 100 μg of triornithine per ml.

The tripeptide concentration used for selection varies according to the medium and strains studied. The resistance of a certain strain to triornithine increases with the complexity of the medium. *E. coli* B, which is a triornithine-sensitive strain (1, 3, 8), is not inhibited by 100 μg of triornithine per ml when grown in nutrient broth or agar. Similar results have been obtained with *E. coli* W and *E. coli* K-12 strains. One should note the high frequency of mutation documented in Table II in order to calculate the number of colonies likely to appear when selecting for TOR on triornithine-containing plates.

All TOR mutants show similar morphology to their parental strains, as judged by light microscopic studies. In addition, the morphology of their colonies on agar plates is the same as that of the wild type.

Characterization of TOR Mutants—Triornithine resistance can be achieved by preventing entrance of the drug, neutralizing the effect of the toxic agent, or by changes of the target site on which the peptide acts. It is therefore necessary to determine the type of change responsible for the TOR phenotype in any given organism. Figs. 1 and 2 compare the typical behavior of TOR mutants and their parental strains when tested for peptide uptake.

The transport of peptides into bacteria can be demonstrated by their biological activity as nutritional sources or as internal toxic agents (7). In the following experiments the peptides Pro-Phe and Pro-Phe-Lys are tested for their ability to support the growth of proline auxotrophs TD-V and TD-V-TOR-5 when

TABLE I

Isolation of TOR mutants

Experiment	*Escherichia coli* strain	Medium	Colonies isolated	TOR mutants	TOR mutants Opp^-
Isolation from liquid culture	K-12 TD-V	VB full	5	4	4
	K-12 Hfr-3000	VB full	4	4	4
	K-12 Hfr-H	VB full	6	5	5
	B	M-9	5	5	
	W[a]	A	1	1	1
Isolation on plates	K-12 TD-V	VB full Sm	195	192	
	B	VB Min	3	2	
	W	A	320	320	

[a] T. E. Fickel, unpublished results.

TABLE II

Frequency of TOR mutants

Populations tested were: A, liquid cultures of cells that have been grown with shaking (150 rpm) at 37° in L-broth; B, liquid cultures of cells that have been grown in VB full medium in similar conditions to those indicated in Population A; C, suspensions of isolated colonies that have been grown for 18 hours at 37° on L-b agar plates; D, suspensions of isolated colonies that have been grown for 40 hours at 37° on VB full Sm agar plate. Total cells were scored after appropriate dilution on VB full plates for Hfr-3000 and on VB full Sm plates for strain TD-V. TOR mutants were selected on the above mentioned plates containing 100 μg of triornithine per ml. The colonies were counted after 42 hours of incubation at 37°.

Bacterium	Population tested	TOR mutants				Total cells			Frequency of TOR mutants
		Number of colonies			Cells/ml	Number of colonies		Cells/ml	
		Undiluted	10^{-1} dilution	10^{-2} dilution		5×10^{-5} dilution	10^{-6} dilution		
TD-V	A			20	2.0×10^4		160	1.6×10^8	1.25×10^{-4}
	A			20	2.0×10^4		63	6.3×10^7	3.2×10^{-4}
	B			10	1.0×10^4		435	4.3×10^8	2.3×10^{-6}
	C		66	7	6.8×10^3		232	2.3×10^8	3.0×10^{-5}
	C	686	51		6.0×10^3	407		2.0×10^8	3.0×10^{-5}
	C		239		2.4×10^4	705		3.5×10^8	6.9×10^{-5}
	C	800	62		7.1×10^3	507		2.5×10^8	2.8×10^{-5}
	C	163	16		1.6×10^3	305		1.5×10^8	1.1×10^{-6}
	D	160	14		1.5×10^3	220		1.1×10^8	1.36×10^{-5}
Hfr-3000	C	30			3.0×10^2	80		4.0×10^7	7.5×10^{-6}
	C	29			2.9×10^2	36		1.8×10^7	5.5×10^{-6}
	C	70			7.0×10^2	128		6.4×10^7	3.1×10^{-6}
	C	42			4.2×10^2	119		6.0×10^7	7.0×10^{-6}

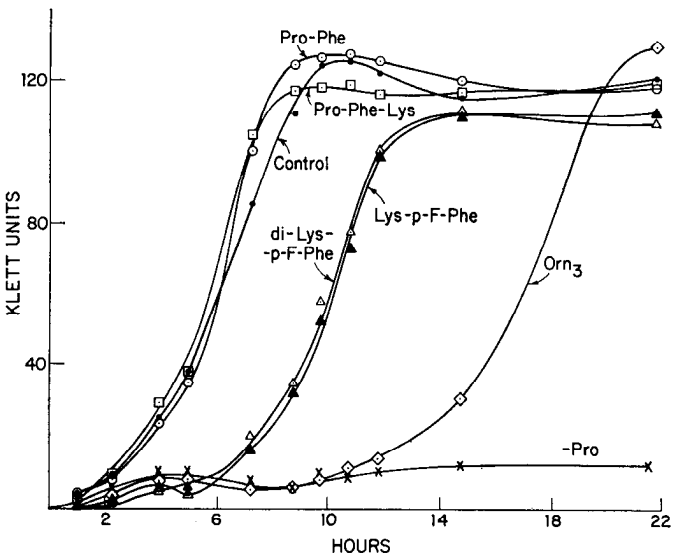

FIG. 1. Peptide uptake in strain TD-V. Growth conditions used are described under "Experimental Procedures." Symbols: ●——●, control growth in VB full Sm; ×——×, growth in VB full Sm void of proline; ◇——◇, VB full Sm + triornithine (100 μg per ml); ▲——▲, VB full Sm + Lys-*p*-F-Phe (90 nM per ml); △——△, VB full Sm + di-Lys-*p*-F-Phe (100 nM per ml); □——□, VB full Sm void of proline + Pro-Phe-Lys (100 μg per ml); ○——○, VB full Sm void of proline + Pro-Phe (70 μg per ml).

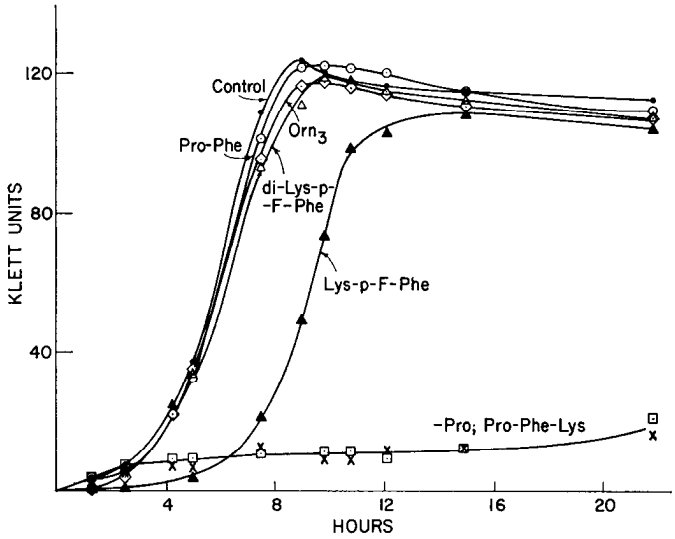

FIG. 2. Peptide uptake in strain TD-V-TOR-5. Growth conditions and symbols used are as described in Fig. 1.

supplied as the sole sources of the essential amino acid. The growth which occurs in the presence of these peptides (Fig. 1) indicates uptake of the di- and tripeptide by the parental strain. Strain TD-V-TOR-5, however, fails to utilize the tripeptide Pro-Phe-Lys for growth, whereas an equimolar concentration of the dipeptide Pro-Phe supports practically normal growth (Fig. 2). These results are best explained by the loss of the oligopeptide transport system but retention of the dipeptide transport system in the TOR mutant. The parental strain also shows sensitivity to the toxic tripeptide tri-L-ornithine, which is known to exert its toxic activity when accumulated intracellularly (2, 3, 5, 7). It is also sensitive to di- and tripeptides containing the toxic amino acid derivative *p*-fluorophenylalanine, such as Lys-*p*-F-Phe and di-Lys-*p*-F-Phe. *p*-Fluorophenylalanine must be integrated into proteins in order to exert its toxic effect (18), and therefore, toxicity requires the penetration of the peptides containing the derivative into the cells. Sensitivity to these toxic peptides can serve, therefore, as an indicator for di- and oligopeptide uptake. The TOR stain is resistant to the toxic tripeptide di-Lys-*p*-F-Phe, whereas its

sensitivity to the dipeptide Lys-*p*-F-Phe and to *p*-F-Phe is similar to the parental strain. This finding further supports the idea that the TOR mutant is blocked in the general oligopeptide transport system, since none of the other TOR possibilities could explain resistance to these two different toxic tripeptides.

This last approach (peptide toxicity) is of special importance when studying peptide transport in wild type organisms in which peptide nutritional utilization cannot be shown easily. The wild types Hfr-H, Hfr-3000, and *E. coli* W were tested and showed the typical sensitivity to the toxic di- and tripeptides, as shown in Fig. 1 for TD-V. Their TOR mutants, however, lost their sensitivity to the toxic tripeptides, triornithine and di-Lys-*p*-F-Phe, and behaved in this respect similarly to TD-V-TOR-5 (Fig. 2).

It should be mentioned here that all the TOR mutants isolated from the three *E. coli* K-12 strains and from *E. coli* W show the same behavior when tested for peptide uptake by utilization, or toxicity of peptides, or both (Table I). It seems therefore that all these TOR mutations are of the same type, *i.e.* oligopeptide permeaseless mutants (Opp^-).

Frequency of TOR Mutants—Previously this laboratory has reported a very high frequency of mutants (5.2×10^{-5}) showing triornithine resistance in an unselected population of *E. coli* W (2). Similar results are obtained with *E. coli* K-12 TD-V (Table II). The frequency of TOR mutants in an unselected population of strain TD-V that has been grown to late log phase, varies from 3×10^{-4} to 2.3×10^{-5} in different experiments. Even suspensions of fresh isolated colonies of this strain that have grown without any selection pressure on rich medium (L-broth) result in a frequency of 1×10^{-5} to 6.9×10^{-5}. The wild type *E. coli* K-12 Hfr-3000 gives a lower frequency of TOR mutants from isolated colonies. However, even these frequencies are high (7×10^{-6} to 1.6×10^{-5}). Moreover, some of the strains of *E. coli* in our laboratory were found to be resistant to triornithine without prior selection. However, the mechanism of resistance in any of these cases has not been studied, and it may not be of the Opp^- nature.

Sensitivity of Parental and TOR Strains to Phages—Mutations at the level of the cell envelope sometimes results in phage resistance. Reciprocally, phage resistance (a cell envelope mutation) has been shown to be sometimes accompanied by an increase in the permeability of antibiotics and colicin to *E. coli* (19, 20). It would be of interest, therefore, to study the sensitivity of TOR strains to several phages in comparison to their parental strains to see if the TOR character was accompanied by changes in the cell envelope.

Sensitivity was tested qualitatively toward ϕ80 vir, P1, T_4, and T_1 phages, in TOR mutants and their parental strains. No changes in sensitivity could be detected in any of the TOR mutants in comparison with the parental strains (Table III). This indicates that no extreme change in the cell envelope accompanies the Opp^- phenotype selected through use of triornithine.

Mapping of TOR Locus by Sexual Recombinations—One of the ways to study the complexity of the oligopeptide transport system in *E. coli* is to map precisely and to compare the location of the *opp*$^-$ mutation in different TOR mutants. We first tried to determine the approximate location of the marker by measuring TOR recombinants after complete mating of the female TD-V with Hfr-3000-TOR strains.

The results obtained showed the appropriate exponential correlation between the rates of recombination and the distances from the F factor for all the known markers of strain TD-V. The marker giving rise to the TOR character was found to be near the *trp* marker. The yield of the mating as judged by the rate of recombination for the closest marker to the origin *leu*, is 40 to 30%. The mating efficiency was found to be the same for TOR and non-TOR organisms.

TABLE III

Sensitivity of Escherichia coli K-12 strains to phages

The sensitivity of the different strains to phages was measured qualitatively as described under "Experimental Procedures."

Strains	ϕ80 vir	P1	T_4	T_1
TD-V (parent)	−	+	+	−
TD-V-TOR-2	−	+	+	−
TD-V-TOR-3	−	+	+	−
TD-V-TOR-4	−	+	+	−
TD-V-TOR-5	−	+	+	−
Hfr-3000 (parent)	+	+	+	+
Hfr-3000-TOR-1	+	+	+	+
Hfr-3000-TOR-2	+	+	+	+
Hfr-3000-TOR-3	+	+	+	+
Hfr-3000-TOR-4	+	+	+	+
Hfr-H-II (parent)	+	+	+	+
Hfr-H-II-TOR-1	+	+	+	+
Hfr-H-III-TOR-1	+	+	+	+
Hfr-H-IV-TOR-1	+	+	+	+
Hfr-H-IV-TOR-2	+	+	+	+

In spite of that, the direct determination of the TOR recombinants after mating with Hfr-3000-TOR strains is difficult and not highly reproducible. These difficulties are due to the very high rate of TOR mutation in the female TD-V (Table II), which results in a high background of TOR colonies, and interferes with the counting of the TOR recombinants. Moreover, TOR recombinants cannot be selected directly on plates containing triornithine, since the existence of remaining active oligopeptide permease in a recombinant can result in the accumulation of the toxic peptide. One must wait, therefore, for several generations until the inactive product of the new genome will replace the old permease. Triornithine is added, therefore, to the plates only after three to four generations.

As indicated above, the TOR locus is found to be near the *trp* marker. The rate of trp^+ recombinants vary from 6.2 to 8.6 per 100 Hfr cells, whereas the TOR recombinants, after subtracting the TOR mutations, are 4.8 to 6.3 per 100 Hfr-TOR cells.

In the next series of experiments, attempts were made to measure linkage of the locus giving rise to the TOR phenotype to any of the known markers of the female. Recombinants were selected for each of these markers and secondarily examined for the presence of the others. Scoring of TOR cells in these kinds of experiments is facilitated since recombinants are allowed to replicate several times in the primary selection enabling them to integrate the newly coded defective permease into their membranes. Thus, one can, in contrast to the previous experiments, use direct selection on plates containing triornithine.

In Table IV evidence is presented showing almost the same rates of recombination for *trp* and TOR markers in any type of selection when mating strain TD-V with any of three Hfr-3000-TOR strains. The appearance of Trp^+ recombination is nearly always accompanied by TOR and *vice versa*. Out of 391 Trp^+ recombinants which have been selected primarily or secondarily after mating with TOR strains, 387 show the TOR phenomenon. TOR cells, however, are not derived entirely by recombination. The original female contains a certain number of TORs and

TABLE IV

Linkage of markers in sexual recombination

The initially selected recombinants were grown on the appropriate plate at 37° for 40 hours. Colonies were then picked at random and placed on a master plate. Replica plating was carried out after 14 hours of incubation at 37°. The secondarily selected recombinants were inspected for growth after 14 hours of incubation at 37°. VB full Sm plates containing 100 μg of triornithine per ml were used for selection of TOR recombinants.

Hfr used	Selected recombinants	No. scored	Percentage of recombinants				
			Leu$^+$	Pro$^+$	Trp$^+$	TOR	His$^+$
Hfr-3000-TOR-1	Leu$^+$	78	100	64	18	18	2.5
Hfr-3000	Leu$^+$	27	100	52	11	7	0
Hfr-3000-TOR-1	Pro$^+$	35		100	20	14	3
Hfr-3000	Pro$^+$	33		100	12	0	6
Hfr-3000-TOR-1	Trp$^+$	57		67	100	100	17.5
Hfr-3000-TOR-2	Trp$^+$	68		56	100	98	21
Hfr-3000-TOR-2	Trp$^+$	47	61	70	100	100	30
Hfr-3000-TOR-4	Trp$^+$	94		64	100	100	29
Hfr-3000	Trp$^+$	98		62	100	5	11
Hfr-3000-TOR-1	TOR	68		67	97	97	9
Hfr-3000-TOR-2	TOR	44		54	100	100	27
Hfr-3000-TOR-4	TOR	59		46	98	100	24
Hfr-3000	TOR	24		37.5	21	100	8
Hfr-3000-TOR-1	His$^+$	105		48	66	66	100
Hfr-3000	His$^+$	92		47	56	0	100

these will persist through the selection procedure as was shown in a control experiment by mating with the wild type. The number of TORs therefore is higher than *trp*$^+$ when selecting from recombinants of a proximal marker. In these cases the probability of TOR recombinants is low and the background of the mutation is noticeable. Out of 22 TOR, 15 were also Trp$^+$ when selecting from Pro$^+$ and Leu$^+$ recombinants. However, when the selected marker is distal to the region containing the *trp* locus and that giving rise to TOR phenotype, the number of TOR is almost equal to Trp$^+$ as confirmed by examining these markers in His$^+$ recombinants. In the total scored recombinants of Hfr-TOR crosses, which have not been selected directly for *trp*$^+$, 285 are TOR, of which 276 are Trp$^+$. The complete linkage between *trp* and TOR, when taking into consideration additional correlations between *trp*$^-$ and sensitivity to triornithine appears to be 666 out of 679. Thus, the linkage between these two markers, as judged by sexual recombination, is 98%.

Using the information of the linkage of TOR to Trp, we have succeeded in curing TOR by episomal transfer from *E. coli* K-12 4256. *E. coli* 4256 is an F′ strain that carries a *trp*$^+$ episome. This episome originated from strain Hfr B_7 (11) and contains therefore genetic information from 30 to at least 27 min of the chromosome. Since we have calculated that the *opp* gene is on the *cysB* side of the *trp* marker (see "Discussion") we were sure that this episome contains the *opp* gene. Episome transfer from this strain to the *trp*$^-$ *opp*$^-$ strain should result therefore, in getting diploids and recombinants which show the *trp*$^+$ *opp*$^+$ character.

Mating mixtures of 4256 with TD-V-TOR-3 and TD-V-TOR-5 were selected for *trp*$^+$ and checked for TOR. Of 118 *trp*$^+$ recombinants checked, 68 originated from mating with TD-V-TOR-3 and 50 from TD-V-TOR-5. The results obtained showed complete sensitivity of all the Trp$^+$ recombinants to triornithine. These findings support the previous conclusion concerning the close linkage of *opp* to *trp*.

TABLE V

Linkage of TOR to trp determined by P1-mediated co-transduction

Trp$^+$ transductants were selected on VB full Sm without tryptophan. After 40 hours of growth at 37°, colonies were picked at random and placed on master plates containing the same medium. Replica plating was carried out with sterile velvet pads after allowing the colonies to grow for 14 hours at 37°. Growth of the TOR transductants was determined after a similar period of incubation at 37°. VB full Sm plates containing 100 μg of triornithine per ml were used for selection of TOR transductants.

Origin of P1 particles	No. of Trp$^+$ scored	TOR co-transductants	
		No.	Percentage
Hfr-3000	28	0	0
Hfr-3000-TOR-1	93	89	95
Hfr-3000-TOR-1	92	90	98
Total	185	179	97
Hfr-3000-TOR-2	21	19	90
Hfr-3000-TOR-2	80	78	97
Hfr-3000-TOR-2	74	73	99
Total	175	170	97
Hfr-3000-TOR-3	34	33	97
Hfr-3000-TOR-3	45	39	87
Hfr-3000-TOR-3	62	56	90
Total	141	128	91
Hfr-3000-TOR-4	17	15	88
Hfr-3000-TOR-4	44	41	93
Total	61	56	92
Hfr-H-II-TOR-1	68	55	81
Hfr-H-II-TOR-1	97	77	79
Total	165	132	80
Hfr-H-III-TOR-1	41	31	75
Hfr-H-III-TOR-1	98	84	83
Total	139	115	83
Hfr-H-III-TOR-2	113	97	86

Fine Mapping of TOR Locus by P1 Transduction—Markers that are very closely linked on the *E. coli* chromosome can be co-transduced because they can be carried into the cell on the same P1 phage particle. In fact, the frequency of co-transduction often serves as the best genetic estimate of the distance between two markers. P1 transduction has been used, therefore, to correlate the TOR marker with *trp*. P1 particles which have been isolated from different TOR mutants (*trp*$^+$ *opp*$^-$) are transduced into strain TD-V (trp$^-$ opp$^+$). Selection is carried out for Trp$^+$ transductants and the frequency of TOR among these transductants is checked by secondary selection. The results of this kind of experiment are shown in Table V. Co-transduction frequencies for these two loci are around 80% and 90 to 95% in Hfr-H-TOR strains and Hfr-3000-TOR strains, respectively. As expected, no co-transduction is found between *pro*$^+$ or *his*$^+$ with the locus causing TOR or between *trp*$^+$ with

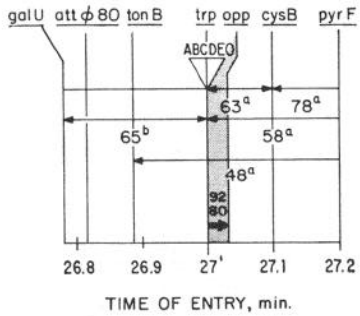

FIG. 3. Genetic map of the *trp* region in *Escherichia coli* K-12 determined by P1 co-transduction. The relative location of the markers is according to Taylor and Trotter (22). The *shaded area* is the one studied in this paper. Frequencies of co-transduction are given as percentages: *a*, Signer *et al.* (25); *b*, Guest (24).

leu, *pro*, *his*, and *arg*, indicating lack of double infections. Moreover, Trp^+ transductants isolated after infection with P1 particles which originated from Hfr-3000 (trp^+ opp^+) does not show any TOR character. Three trp^+ TOR transductants were picked to be tested for peptide uptake. All three showed a lack of oligopeptide transport but normal dipeptide transport as judged by nutritional response and toxicity of the peptides indicated above (see Fig. 1). These findings support the idea that TOR is a specific phenotypic expression of the opp^- genotype.

DISCUSSION

All our 12 new TOR mutants which have been isolated from several strains of *E. coli*, like all the previously reported TOR strains, are found to be defective in their oligopeptide transport system. None of the other theoretical possibilities to achieve triornithine resistance in *E. coli* is detected. Moreover, all of these TOR mutants occur at the same region near *trp* without any significant differences in location. It seems, therefore, plausible that all the presently isolated TOR mutants are altered in the same gene or operon. The failure to detect the other postulated types of TOR mutants might be due to the very high frequency of mutation at the *opp* locus, which is approximately 100-fold higher than the normal frequency of mutation in *E. coli*. The excess of these mutants covers up all the other types of TOR mutation. It might also be possible that all the other TOR mutations are lethal and cannot exist under normal growth conditions.

Mutation resulting in a change of the cell envelope, such as phage resistance, often affects the permeability of certain components like iron, antibiotic drugs, and colicins to *E. coli* cells (19–21). One might argue, therefore, that the basis of Opp^- phenotype is a general change in the cell envelope. This possibility is ruled out by the following observations: (*a*) all the TOR mutants show the same phage sensitivity as their parental strains; (*b*) no morphological changes in the cells or the colonies of the mutants can be detected; (*c*) the rate of growth of the mutants is similar to their parental strains; (*d*) the dipeptide transport system is not affected by TOR mutations. All of these findings support the idea that TOR strains are single gene mutants with lesions in the gene (or genes) that is responsible for the oligopeptide transport system. The mapping of this gene further supports this conclusion, since all the TOR mutants, even those which originated from different strains of *E. coli* K-12 have mutations in the same region. Co-transduction experiments revealed that the *opp* gene is very closely linked to *trp*. It is the closest gene to *trp* as judged by frequency of co-transduction (Fig. 3). The *opp* gene was found not to be located at the *att φ80-tonB* side of the *trp* operon, since strain TD-V is an Opp^+ strain which does not contain any of the region between *trp* to *att φ80*. The region giving rise to TOR might represent, however, an operon that contains several genes that are essential for the oligopeptide transport. The number of colonies scored for co-transduction is not high enough to indicate different location of the TOR mutations within this region in the strains studied. Complementation experiments using episomes or φ80-*opp* from the different TOR strains should provide the answer concerning the complexity of the oligopeptide transport locus.

The specific location of the *opp* gene (or genes) near *trp* provides a finding of great potential utility since this locus is near the attachment site of phage φ80. One can construct an HFT phage line φ80-*opp* which might yield after induction, a culture synthesizing large amounts of the gene product (25), and might serve, therefore, in the isolation of the oligopeptide permease.

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