JOURNAL OF BACTERIOLOGY, Feb. 1974, p. 502-508
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Vol. 117, No. 2
Printed in U.S.A.

Localization of Two Functions of the Phosphoribosyl Anthranilate Transferase of *Escherichia coli* to Distinct Regions of the Polypeptide Chain

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Received for publication 19 October 1973

The *trpD* gene specifies a polypeptide which has both glutamine amidotransferase and phosphoribosyl anthranilate (PRA) transferase activities. Deletions fusing segments of *trpD* to the gene preceding it in the operon, *trpE*, were selected in strains carrying various *trpD* point mutations. The selection procedure required both that a deletion enter *trpE* and that it restore the PRA transferase activity which the parent *trpD* point mutant lacked. Deletion mutants were found which had PRA transferase activity although the first third of *trpD* was deleted. The existence of the mutants proves that a terminal segment of *trpD* is sufficient to specify a polypeptide having PRA transferase activity. The location of the deletion end points on the genetic map of *trpD* defines the extent of the *trpD* segment required for PRA transferase activity. This segment did not overlap the initial region of *trpD* required to specify the glutamine amidotransferase function of the *trpD* polypeptide. These results support the hypothesis (M. Grieshaber and R. Bauerle, 1972; H. Zalkin and L. H. Hwang, 1971) that the bifunctional *trpD* polypeptide might have evolved by fusion of a gene specifying a glutamine amidotransferase with a gene directing PRA transferase synthesis.

The operator proximal structural genes in the tryptophan operon of *Escherichia coli*, *trpE* and *trpD* (see Fig. 1), specify single polypeptide chains which associate to form the anthranilate synthetase-phosphoribosyl anthranilate (PRA) transferase complex (5, 6, 18). This complex catalyzes the first two reactions unique to tryptophan biosynthesis: (i) chorismate + L-glutamine → anthranilate + L-glutamate + pyruvate; and (ii) anthranilate + 5-phosphoribosyl-1-pyrophosphate → N-5'-PRA + PP_i. The anthranilate synthetase-PRA transferase complex is a tetramer containing two molecules of the *trpE* polypeptide (designated component I) and two molecules of the *trpD* polypeptide (designated component II). In the absence of component II, component I is inactive in the conversion of chorismate to anthranilate with glutamine as amino donor, but will catalyze the synthesis of anthranilate from chorismate when NH_3 replaces glutamine as amino donor, as shown in equation (iii): chorismate + NH_3 → anthranilate + pyruvate. Free component II is as active as the complex in reaction (ii) (the PRA transferase reaction). In addition, component II contributes the glutamine amidotransferase function of the complex, thus permitting the anthranilate synthetase complex to use glutamine as well as NH_3 as amino donor in anthranilate synthesis (5, 6, 12, 18). Thus component II, the *trpD* polypeptide, has two enzymatic activities.

The properties of *trpD* mutants led to the suggestion that the two activities of component II belong to two nonoverlapping regions of the molecule (18). Mutants with alterations located in the initial segment of *trpD* do not accumulate anthranilate. Extracts of these mutants contain component I but lack glutamine amidotransferase activity; that is, they cannot perform reaction (i). In contrast, mutants with alterations in the last two-thirds of the gene accumulate anthranilate, and extracts of these mutants can perform the glutamine-dependent synthesis of anthranilate. Mutants in which the operator-distal two-thirds of *trpD* is deleted also accumulate anthranilate and yield extracts which can catalyze reaction (i). These findings suggest that about the first one-third of the *trpD* polypeptide is sufficient to permit utilization of glutamine in the anthranilate synthetase reaction. This hypothesis is supported by studies of the structurally similar anthranilate synthetase-PRA transferase complex of *Salmonella*

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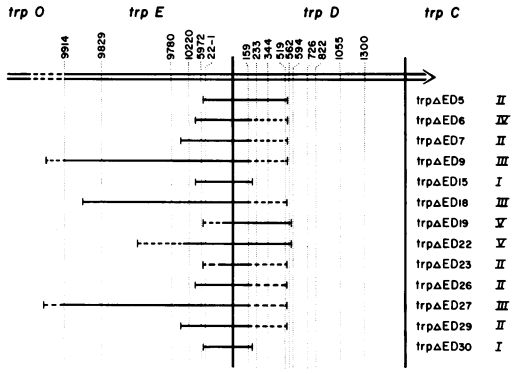

FIG. 1. *Deletions which restore PRA transferase activity. The map shows the first two of the five genes of the tryptophan operon. The lengths of the genes and the location of point mutant sites within each gene are to scale (18). A horizontal bar on the map represents the extent of the deletion. A dotted extension to a horizontal bar indicates that the extent of the deletion in that region of the map has not been defined. A vertical bar at the end of a dotted extension defines the maximal extent of the deletion. All deletions shown are independent isolates. The parent strain from which each deletion was derived is indicated by a Roman numeral (see Table 1). The numbers assigned to trpD mutants are presumed to correspond approximately to the base pair in the gene altered by mutation, with the numbering beginning with the nucleotide corresponding to the first base in the translated region of the message (18).*

typhimurium. Grieshaber and Bauerle (3) and Hwang and Zalkin (4) found that trypsin action on the *S. typhimurium* component II (3) or on the anthranilate synthetase-PRA transferase complex (4) destroyed the PRA transferase activity of component II but yielded a polypeptide fragment, presumably the amino terminal third of component II, which had full activity in promoting glutamine utilization in the anthranilate synthetase reaction.

Conversely, PRA transferase activity may reside only in the last two-thirds of the *trpD* polypeptide. This suggestion is supported by the finding that some polar mutants having chain termination sites early in *trpD* are leaky (grow slowly on minimal medium) and have low but measurable levels of PRA transferase activity (18). The low level of activity could be explained if polypeptide synthesis were reinitiated after the chain termination codon and if the terminal two-thirds of the *trpD* polypeptide were sufficient for PRA transferase activity.

Zalkin and Hwang (19) and Grieshaber and Bauerle (3) suggest that two independent regions of the polypeptide contribute the two activities of component II and that this bifunctional polypeptide might have arisen by fusion of a gene for a glutamine amidotransferase with the gene for PRA transferase. This hypothesis is congruent with the observation in *Serratia marcescens* (19), *Bacillus subtilis* (8), and various strains of *Pseudomonas* (13) that the glutamine-utilizing activity of the anthranilate synthetase complex is contributed by a small polypeptide approximately one-third the size of *E. coli* component II. The PRA transferase of these organisms is not associated with this peptide or with the anthranilate synthetase complex.

We were interested in determining whether, in fact, the terminal two-thirds of *trpD* carries all of the information needed to specify a polypeptide having PRA transferase activity. We reasoned that, if this were the case, in-phase fusions of this segment of *trpD* to the preceding gene in the *trp* operon would yield active PRA transferase.

MATERIALS AND METHODS

Bacterial strains. Derivatives of *E. coli* K-12 strain W3110 were used throughout (See Table 1). The characterization and mapping of the various *trp* operon point mutations are described elsewhere (18). The *trpR*$^-$ allele results in constitutive synthesis of all *trp* operon enzymes when the cells are grown in the presence of excess tryptophan. *tna*$^-$ cells lack a functional tryptophanase (1). The *cysB*$^+$ *trpE*$^+$*D159amC*$^+$*B*$^+$*A*$^+$ *colVB* episome used is a derivative of the Fredericq episome (P. Fredericq, Proc.

TABLE 1. *Strains examined for production of trp deletion mutants which grow on anthranilate*[a]

Strain	Ant$^+$ mutants with deletions (%)	Parent designation
trpR$^+$ *trpD159oc*	8	I
trpR$^-$ *trpD159oc*	20	II
trpR$^-$ *trpE9829am trpD159oc*	6	III
trpR$^-$ *trpD519fs*	0.2	IV
trpR$^-$ *tnal*$^-$ *trpD562ms*	1	V
trpR$^-$ *trpD726fs*	0	
trpR$^-$ *trpD822fs*	0	
trpR$^-$ *trpD1055oc*	0	

[a] Ant$^+$ mutants grow on minimal medium supplemented with anthranilate. Abbreviations: *oc*, ochre; *am*, amber; *fs*, frameshift; and *ms*, missense mutation. The characterization and mapping of these *trp* operon point mutations is described elsewhere (18). The *trpD* point mutant strains listed in the left-hand column do not grow on minimal medium supplemented with anthranilate. Ant$^+$ derivatives were selected, and those cases in which a deletion was the mutational event resulting in the Ant$^+$ phenotype were scored as described in Materials and Methods.

11th Intern. Congr. Genet. 1:42-43, 1963) into which the *trpD159am* point mutation was introduced.

Media. The minimal medium of Vogel and Bonner was used (17). For solid media, it was supplemented with 0.2% glucose and 1.5% agar. Anthranilate agar contained, in addition, 3 μg of anthranilate per ml. Assay medium was liquid minimal medium supplemented with 0.5% glucose, 50 μg of L-tryptophan per ml, 100 μg each of L-tyrosine and L-phenylalanine per ml, and 1 μg each of *p*-aminobenzoic acid and *p*-hydroxybenzoic acid per ml.

Isolation of deletion mutants. Each of the strains listed in Table 1 carries a chain-termination, frameshift, or missense mutation in *trpD*. These mutants do not grow on minimal medium supplemented with anthranilate because they lack PRA transferase activity. From these strains we selected spontaneously occurring derivatives which were able to grow on minimal medium containing anthranilate by plating 5×10^7 cells of one of the *trpD* point mutant parent strains on anthranilate agar. This procedure yielded Trp^- colonies able to grow on anthranilate (Ant^+ phenotype). Those cases in which the ability to grow on anthranilate agar had been restored by a deletion event fusing part of *trpD* to part of *trpE* were detected by replicating Trp^- Ant^+ colonies to medium lacking tryptophan which had been spread with a *ϕ*80 transducing phage able to transduce the parental strain to Trp^+. Derivatives of parents I, II, and IV were replicated to *ϕ80hptEAtrpE5972*, which carries an intact *trp* operon having a point mutation late in *trpE*. If the Trp^- Ant^+ mutant carries a deletion which covers part of *trpD* and extends into *trpE*, no Trp^+ transductants will be found. Ant^+ mutants of parent III were replicated to a *ϕ80h* derivative which carries an ochre suppressor. If a deletion has occurred in the *trpEam trpDoc* parent III, the resulting Trp^- Ant^+ mutant will not be suppressed to Trp^+ by the introduction of the ochre suppressor. For parent V, all Trp^- Ant^+ mutants which appeared were assumed to be deletions. Derivation of the transducing phage and details of the replication procedure are described elsewhere (7). The deletions were mapped by recombination with trp^- point mutants as described elsewhere (7).

Enzyme assays. Except as noted in the legend for Table 3, cultures were grown for enzyme assay in assay medium, which contains excess tryptophan, and harvested during the exponential phase of growth. The preparation of cell extracts and assays of PRA transferase, tryptophan synthetase, and anthranilate synthetase (glutamine as amino donor) are described elsewhere (7). When anthranilate synthetase activity was assayed with NH_3 as amino donor, the reaction mixture contained 10 mM magnesium acetate, 48 mM ammonium sulfate, 50 mM triethanolamine-HCl (pH 8.9), and 1 mM dithiothreitol. This assay mixture is a slight modification of that used by Zalkin and Kling (20).

RESULTS

Fusions of trpE and trpD. We devised a simple procedure which enabled us to select deletions of the first one-third of *trpD* which fuse the remaining *trpD* segment to various segments of *trpE*. The selection procedure required that the fusion events restore PRA transferase activity to mutants which lack it. The deletions were selected in strains having a point mutation early in *trpD*. These strains have little or no PRA transferase activity and thus fail to grow on minimal medium supplemented with anthranilate. Derivatives of these strains which would grow on anthranilate were selected. Since PRA transferase is required to synthesize tryptophan from anthranilate, the mutants which grow on anthranilate must have acquired PRA transferase activity. In some cases, a deletion fusing *trpD* and *trpE* segments was the mutational event which restored PRA transferase activity. These cases were identified initially by testing for loss of *trpE* function and verified by transduction analyses with *trpE* point mutants. Table 1 lists the *trpD* point mutant strains in which anthranilate-responding deletion mutants were sought. The position of the point mutations on the genetic map of *trpD* is shown in Fig. 1. As seen in Table 1, anthranilate-responding deletion mutants were readily obtained in parental strains carrying *trpD* nonsense, frameshift, or missense mutations at positions 159 through 562 but not in *trpD* mutants altered in more distal regions of the gene. The extent of some of these deletions is shown on the map in Fig. 1. The level of PRA transferase activity measured in extracts of these deletion strains is shown in Table 2. The deletion strains all lacked an initial segment of *trpD* but had PRA transferase activity. These findings prove that a terminal segment of *trpD* is sufficient to specify a polypeptide having PRA transferase activity.

The deletion map (Fig. 1) allows fairly precise definition of the extent of the *trpD* segment required for PRA transferase activity. Deletions *trpΔED22* and *trpΔED19* removed the initial portion of *trpD* through approximately position 562 (see Fig. 1), but the strains bearing these deletions had PRA transferase activity and grew on minimal medium supplemented with anthranilate. Therefore, the left-hand end (see Fig. 1) of the minimal segment of *trpD* required to specify PRA transferase lies to the right of *trpD562*. It is interesting that the strain bearing *trpD562* is a missense mutant which has full activity in stimulating glutamine utilization by component I and accumulates anthranilate because it lacks active PRA transferase (18). It appears that deletions of the *trpD562* site restore the PRA transferase activity that was abolished by an amino acid substitution at the

TABLE 2. *Specific activities of trp operon enzymes in trp deletion mutants which grow on anthranilate*[a]

Deletion strain	Sp act[b]		Polarity[c]
	PRA transferase	TSase β_2 or α	
trpΔED5	0.22	17.0	~100
trpΔED6	0.14	19.0	~100
trpΔED7	0.01	14.0	96
trpΔED9	Trace	5.6	41
trpΔED15	0.007	3.5	24
trpΔED18	0.13	13.3	~100
trpΔED19	0.009	12.3	90
trpΔED22	0.009	15.8	~100
trpΔED23	0.46	22.0	~100
trpΔED26	0.02	3.5	16
trpΔED27	0.15	12.6	93
trpΔED29	0.02	3.0	23
trpΔED30	0.005	3.1	23
W3110 *trp*$^+$	1.2-1.7	11-19	

[a] All strains in the Table carry a *trpR*$^-$ allele which results in constitutive synthesis of all *trp* operon enzymes when the cells are grown in the presence of excess tryptophan. These strains were grown for enzyme assay in assay medium containing excess tryptophan, harvested in the exponential phase of growth, sonicated, and assayed as described in Materials and Methods. Percentage of wild-type activity was calculated relative to a wild-type culture (W3110 *trpR*$^-$*trp*$^+$) grown in parallel at the same time. None of the deletion strains in the Table had detectable anthranilate synthetase activity with either glutamine or NH_3 as amino donor.

[b] Expressed as units of enzyme activity per milligram of protein.

[c] Expressed as percentage of tryptophan synthetase β_2 or α activity of control culture.

trpD562 site. The finding that the *trpD562* site is not necessary to specify an active PRA transferase polypeptide resolves what had previously seemed an anomaly. The finding that *trpD562* is not leaky was interpreted to indicate that its amino acid substitution was in a region of the PRA transferase polypeptide essential for activity. Yet frameshift mutant *trpD594*, whose alteration lies to the right of *trpD562*, is leaky, suggesting that a site beyond that of *trpD562*, namely the *trpD594* site, is not within the *trpD* segment necessary to specify a polypeptide having PRA transferase activity (18). The present result, that the *trpD562* site is not essential for PRA transferase activity, resolves the earlier paradox and, together with the properties of *trpD594*, allows the conclusion that the left-hand end of the PRA transferase segment of the *trpD* polypeptide probably lies to the right of the *trpD594* site.

It has previously been shown by point mutant and deletion analyses that the end of the region of *trpD* needed to provide the glutamine amidotransferase function of component II lies between *trpD519* and *trpD562* (18). It is now clear that there is no overlap of the two segments of *trpD* necessary to specify the two activities of component II. This conclusion is in accord with the above definition of the termini of the two segments. In addition, the *trpD594* mutation results in the production of low levels of both glutamine-dependent anthranilate synthetase activity and PRA transferase activity (18). The point mutation in this strain must lie between the two segments of *trpD*, in a region which is not essential to specify either activity.

Phase of deletions. If deletions joining part of *trpD* with part of *trpE* occur randomly, two of every three deletions should result in a fusion which is not in the proper reading frame for correct translation of the *trpD* segment specifying PRA transferase. However, such strains could have PRA transferase activity if the consequence of the deletion is to place a chain termination site in the vicinity of a restart site (14). Would these out-of-phase fusions be found in our set of deletion mutants? The data in Table 2 show that a number of deletion mutants have less than the wild-type amount of tryptophan synthetase β_2 or α. These enzymes are specified by genes in the *trp* operon located operator-distal to *trpD*. An out-of-phase deletion early in *trpD* should generate a chain termination codon in the new reading frame near the deletion terminus and could therefore exert a polar effect on synthesis of polypeptides specified by distal genes in the operon. Thus, the low levels of tryptophan synthetase β_2 or α in some deletion mutants indicate that those strains carry out-of-phase deletions (7). In these strains, the PRA transferase activity may result from reinitiation of translation in the proper reading frame at a site near the chain termination codon generated by the deletion, yielding a polypeptide fragment of component II. Conversely, those strains which synthesize as much tryptophan synthetase β_2 and α as wild type probably have in-phase deletions. In most of these mutant strains, a fused protein is presumably synthesized which contains some *trpE* sequences fused to the *trpD* polypeptide segment which has PRA transferase activity. It is conceivable, however, that occasionally an out-of-phase deletion might place a chain-termination codon immediately adjacent to an efficient restart site and that no polar effect would be generated on the distal genes of the operon. An analogous example has been described in the

galactose operon of *E. coli* (15). However, such deletions should be rare. It is most probable that the collection of mutants listed in Table 2 includes both in-phase and out-of-phase deletions.

Eight of the deletion strains listed in Table 2 have nonpolar levels of tryptophan synthetase (>90% of the Trp^+ level) and thus probably carry in-phase fusions. In these strains, the remaining *trpE* polypeptide fragment and the PRA transferase fragment probably are synthesized as a single polypeptide chain. Since these eight deletions have at least six different left-hand end points in *trpE*, the PRA transferase activity must be relatively independent of the amino acid sequence in the portion of the polypeptide chain N-terminal to the PRA transferase region.

PRA transferase activity in the deletion mutants studied is unstable in crude extracts. Extracts of deletion strains *trpΔED5* and *trpΔED30* lose 30% of the initial PRA transferase activity in 10 h at 0 C, whereas extracts of mutant *trpΔED15* lose 50% of the initial activity under the same conditions. This instability, superimposed on the initial low levels of PRA transferase activity in most of these strains, prevented us from purifying and characterizing the mutant proteins. However, it is evident from the PRA transferase-specific activities of extracts of some of the deletion strains (Table 2) that the rescued PRA transferase sequence may have appreciable activity compared with the wild-type enzyme.

Is glutamine utilization essential for growth? Previously, it was not possible to establish unequivocally whether the NH_3-dependent activity of component I alone could produce enough anthranilate to support growth of *E. coli* on minimal medium, since it was not possible to eliminate in vivo the glutamine amidotransferase activity of component II without simultaneously eliminating PRA transferase activity. We were able to address this question by using the deletion strains with PRA transferase activity but no glutamine-activating activity. We introduced a functional component I into these strains by transferring in an episome which carries $trpE^+$ and $trpD^-$ markers in the *trp* operon. The *trpD* mutation used, *trpD159*, is strongly polar and eliminates component II glutamine amidotransferase and PRA transferase activities. The properties of the diploids obtained are shown in Table 3. They have no glutamine-dependent anthranilate synthetase activity, but nevertheless they grow well on minimal medium. Thus component I alone can synthesize enough anthranilate by using NH_3 as amino donor to support growth of *E. coli* on minimal medium. This conclusion had been suggested previously by extrapolation of in vitro data (2, 8, 9). This finding explains the earlier observation (18) that all mutants which have lesions in the initial portion of *trpD* and cannot provide glutamine utilization for component I are chain-termination or frameshift, rather than missense, mutations. Our observations with the diploid strains suggest that a missense mutation in this region would not be detected as a Trp^- mutation. The early *trpD* chain-termination and frameshift mutations are Trp^- because they fail to synthesize the distal portion of the polypeptide required for PRA transferase activity. The reason some of these mutants are leaky is now clear. The leaky mutants are known to produce low levels of PRA transferase activity (18). In addition, we now know from the diploid results (Table 3) that enough anthranilate to support growth can be synthesized by component I using NH_3 as amino donor.

DISCUSSION

It has previously been shown that the amino-terminal third of the *trpD* polypeptide (anthranilate synthetase component II), in the presence of the *trpE* polypeptide (anthranilate synthetase component I), is sufficient to permit glutamine utilization in anthranilate synthesis (3, 4,

TABLE 3. *Specific activities of PRA transferase and anthranilate synthetase in deletion strains carrying a $trpE^+D^-amC^+B^+A^+$ episome*[a]

Strain	Sp act			Growth on minimal medium
	PRA transferase	ASase[b] (Gln)	ASase[c] (NH_3)	
$cysB^-$ $trp\Delta ED15/colVB$ $cysB^+$ trpD159	0.035	0	0.172	+
$cysB^-$ $trp\Delta ED5/colVB$ $cysB^+$ trpD159	0.60	0	0.022	+

[a] The Fredericq *$cysB^+$ $trpE^+D159amC^+B^+A^+$ colVB* episome was introduced into the *$trpR^+$ $cysB^-$ trpED* deletion strains by selecting for *$cysB^+$*. The *trpD159* allele is not leaky, and strains haploid for this *trpD* allele have no detectable PRA transferase activity. The diploid strains produced colonies on minimal plates that were as large as those of a wild-type control when scored after 2 days at 37 C. The diploid strains were grown for enzyme assay overnight in minimal medium plus indole (1.5 μg/ml). Preparation of extracts and enzyme assay procedures were as described in Materials and Methods.

[b] Anthranilate synthetase activity with glutamine as amino donor.

[c] Anthranilate synthetase activity with NH_3 as amino donor.

18). Here we have shown that the carboxy-terminal two-thirds of the *trpD* polypeptide is sufficient to catalyze the PRA transferase reaction. Our findings further indicate that the two activities of component II are associated with two distinct, nonoverlapping regions of the polypeptide chain. However, since our studies were qualitative in nature, the possibility remains that interactions between the two regions of the *trpD* polypeptide enhance one or both activities.

The method used to detect deletions resulting in functional gene fusions should be generally applicable to many polygenic operons. Although the deletions which generated PRA transferase activity by fusing segments of *trpD* and *trpE* occurred infrequently, they were readily detected by scoring for loss of *trpE* function concomitant with restoration of PRA transferase activity in a *trpD* mutant. This procedure is formally analogous to the detection of deletion events by scoring for loss of functions controlled by neighboring genes (e.g., reference 16).

Our results support the hypothesis that component II might have evolved by fusion of a gene specifying a glutamine amidotransferase with a gene directing PRA transferase synthesis (3, 19). The *trpD* polypeptide of *E. coli* is analogous to the two separate polypeptides, one providing glutamine amidotransferase activity and the other PRA transferase activity, which are found in some bacterial species (8, 13, 19). Our results suggest that the PRA transferase polypeptide segment of component II can be fused to other polypeptide sequences and retain PRA transferase activity. The possibility that an ancestor of *E. coli* may not have had a glutamine-binding protein associated with anthranilate synthetase is plausible in view of our finding that in vivo component I can produce enough anthranilate for tryptophan synthesis by using NH_3 as a source of the amino group of anthranilate. Recent studies have shown that the amino acid sequences of the amino-terminal segments of the *trpD* polypeptide of *E. coli* and the glutamine amidotransferase polypeptide of the anthranilate synthetase of *Serratia marcescens* are homologous (S. L. Li, J. Hanlon, and C. Yanofsky, unpublished data). Thus, these polypeptides must have had a common ancestral polypeptide.

Margolin and Bauerle earlier reported two mutants of *S. typhimurium* able to utilize anthranilate which have deletions entering the *trp* operon from the operator end and terminating early in the PRA transferase gene (11). Thus, as is the case for *E. coli*, a fragment of the normal *S. typhimurium* polypeptide can have PRA transferase activity.

La Scolea and Balbinder (10) have recently reported that, in *S. typhimurium*, deletions of the initial segment of the gene specifying PRA transferase may restore this activity to mutants which lack it. It was also shown that some of the deletions terminate in the structural gene for component I of the anthranilate synthetase-PRA transferase complex, as observed in the present study. The *S. typhimurium* deletion mutants were studied with the objective of explaining the pleiotrophic properties of one strain having a mutation within the so-called "unusual region" of the *trp* operon of this organism. A mutant comparable to this has not yet been encountered in *E. coli*.

ACKNOWLEDGMENTS

We are grateful to Virginia Horn and Miriam Bonner for their help.

This work was supported by grants from the National Science Foundation (GB6790), Public Health Service (GM-09738 from the National Institute of General Medical Sciences), and the American Heart Association.

E. N. J. is a predoctoral trainee of the Public Health Service. C. Y. is a career investigator of the American Heart Association.

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