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Excretion of Enterochelin by *exbA* and *exbB* Mutants of *Escherichia coli*

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Escherichia coli mutants that are insensitive to colicins B and I hyperproduce and excrete the iron chelator enterochelin, which is an inhibitor of these colicins. These mutants are classified as *exbA* and *exbB*. The *exbA* mutants are chromium sensitive and require iron for growth, and the mutations are located in the *tonB* region at min 25 of the *E. coli* chromosome. *tonB* mutants in which the genome of phage lambda is inserted into the bacterial chromosome within the *tonB* gene also exhibit enterochelin excretion. The *exbB* mutants require methionine and probably result from deletions which are located between min 56 and 58. Colicin insensitivity, enterochelin excretion and methionine auxotrophy are recessive in *exbB* merodiploids. The methionine requirement of *exbB* strains is satisfied by cystathionine or homocysteine, and *exbB* mutants are sensitive to ethionine.

Colicin B- and I-insensitive mutants were described by Gratia (6). Some of the mutations conferred resistance to phage T1, were mapped in the *tonB* gene near the tryptophan operon, and were frequently deletions (7). The *tonB* gene has recently been involved in iron transport: *tonB* mutants are chromium sensitive (16) and show decreased binding constants for iron in kinetic studies of iron transport (17). Gratia (6) described another class of mutants insensitive to colicins B and I which required methionine. The corresponding mutation was located in the section of the *E. coli* chromosome between *his* at min 39 and *str* at min 65.

We present evidence concerning the excretion of enterochelin by a series of newly isolated mutants insensitive to colicins B and I, as well as the physiology of these mutants and the location of the corresponding genes.

MATERIALS AND METHODS

Bacteria and phage. Bacterial strains are listed in Table 1 or have been described previously (8). Phage T5 and T6 were obtained from S. E. Luria; ϕ80*vir*, from E. R. Signer; λ*cI*857 and λ*cI*60, from R. Sussman; P1 and R17, from R. Schleif. Isolation of *exb* mutants from colicin-sensitive strains has been described (8).

Media. OM minimal medium contains per liter of distilled water: 10.5 g of K_2HPO_4; 4.5 g of KH_2PO_4; 0.05 g of $MgSO_4$; 1.0 g of $(NH_4)_2SO_4$, and 1.6 mM sodium citrate. Other media have been described (8). Minimal media were supplemented with 0.2% glucose and 1 μg of thiamine per ml.

Phage P1 was titered on LB agar with 2.5×10^{-4} M $CaCl_2$ and 0.1% glucose. Chromium sensitivity was determined on LB agar supplemented with 4×10^{-4} M $CrCl_3$.

Colicin techniques. Preparation and titration of colicin B and colicin inhibitor have been described (8).

Bacterial conjugation. Matings were performed according to Nagel de Zwaig and Luria (12). A *recA* derivative of strain LD60 *exbB metL serA tsx* was constructed by conjugation with Hfr 111 *recA nalB* and selection for recombinants resistant to T6 and to nalidixic acid and the *recA* phenotype (sensitivity to ultraviolet light or methylmethane sulfonate). Into one such recombinant, LD63 *recA nalB exbB serA metL tsx*, the episome F' KLF16 containing genes from *metC* to *fuc* was introduced by mating with strain KL110/KLF16, and merodiploids were selected by plating on minimal agar with methionine. The exconjugants that grew were the merodiploids LD63/KLF16.

Transductions. Recipient cells for P1 transductions were grown in LB broth to log phase, and $CaCl_2$ was added to 5×10^{-3} M. Phage P1 grown on the appropriate donor was added at a multiplicity of 0.1 to 0.2 phage per cell. After 15 min at 37 C, samples of the mixture or controls were plated on selective media.

Selection of λ-induced tonB mutants. Log-phase cells of strain B(583) Δ24 (*gal bio* λ*att*) were infected with λ*cI*857 (temperature inducible) and grown 4 h at

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TABLE 1. *Bacterial strains, E. coli K-12*

Strain	Relevant characteristics	Source[a]
GUC6	*exbA*[b] from C600	this paper
GUC12	*exbA* from C600	this paper
GUC41	*exbB metL* from C600	this paper
GUC49	colicin B insensitive, from C600	this paper
C600	*thr leu tonA*	L104
W4032	*pro met tsx* Hfr Cav	LA235
GUW5	*exbA* from W4032	this paper
PA309	*thr leu trp his arg thi str* F^-	LA435
WA5028a	*trpA* double-point mutant	E. Signer
X5050	*lac-pro* deletion (ϕ80d*lac*)	E. Signer
VXII	deletion from *trp* to *lac* in prophage, from X5050	E. Signer
M107	suI	E. Signer
B(583) Δ24	*gal* λ*att bio* deletion	D. Freifelder
3LH9	*tonB* (λ*cI*857) from B(583) Δ24	this paper
P10	*thr leu malB lac* str^R Hfr	C. Kennedy
LD28	*exbB metL* from P10	this paper
A2325	*argE his thi pro trp mtl gal tsx* F^-	D. Boyd
LD54	*exbB metL* from A2325	this paper
KL16	Hfr	LA761
AB856	*serA6*	B. Bachmann
LD60	*serA exbB metL tsx* from AB856	this paper
LD63	*serA exbB metL recA nalB tsx* from LD60 and Hfr 111	this paper
Hfr 111	*recA nalB*	R. Schleif
KL110/KLF16	chromosome: *argG6 metB1 his*-1 *thy*-23 *leu*-6 *recA1*; F': from AB312, $metC^+$ to fuc^+	B. Bachmann

[a] Symbols refer to Luria stock collection.

[b] Genetic abbreviations are described (15) or defined in this paper.

30 C. Samples were cospread with sufficient colicin B, ϕ80*vir* and λ*cI*60 so that, on control plates with any one of these three agents, most of the cells were killed. Surviving colonies were picked to duplicate grids on LB plates which were incubated at 30 or 42 C. Twelve ts isolates were obtained, three of which produced phage lambda and were considered to be *tonB* (λ*cI*857).

High-titer λ for transduction was prepared from one such strain 3LH9 and tested for ability to transduce Trp^+ to strain WA5028a *trpA*. Transductants appeared at a frequency of 3×10^{-5}.

RESULTS

Isolation and phenotype of exb mutants. Independent spontaneous colicin-insensitive mutants of *E. coli* K-12 strain C600 were obtained (8) by selection with colicin produced by strains C1 139 (Col B, M), CA53 (Col Ia), C600 (Col Ib), or K94 (Col V). Mutants fell into various classes: insensitive to colicin B, I, or V; or to B and I, I and V, and B and V; or to all three colicins B, I, and V. Response of mutants to colicins Ia and Ib was identical. Some of these excrete large quantities of enterochelin, an inhibitor of colicins B and I, and have been named *exb* mutants (8). Some of these mutants have a requirement for methionine.

Enterochelin production in wild-type strains is repressed by exogenous iron (3,18). In *exb* strains, enterochelin is produced in high quantities in media containing up to 2 mM $FeCl_3$. Of the two classes of *exb* mutants distinguished genetically below, the *exbA* type produces a larger quantity of colicin inhibitor than the *exbB* (Fig. 1). For example, GUC6 *exbA* and GUC41 *exbB* produced 1,000- and 250-fold more enterochelin, respectively, than strain C600, as judged by titration of culture supernatant fluids against colicin B (8).

Map location of the exbA mutation. Strain GUW5*exb* was isolated from strain W4032 Hfr Cav *pro met* and crossed with the multiauxotrophic recipient PA309 (Table 2). The results, although not fully consistent, suggested that the *exb* gene might be most close to the *trp* locus. This in turn suggested that some *exb* mutants might map in the *tonB* gene, adjacent to the *trp* operon (15).

The *tonB* phenotype, resistance to phages T1 and ϕ80, cannot be observed in mutants of C600, a *tonA* strain resistant to T1, T5, and ϕ80.

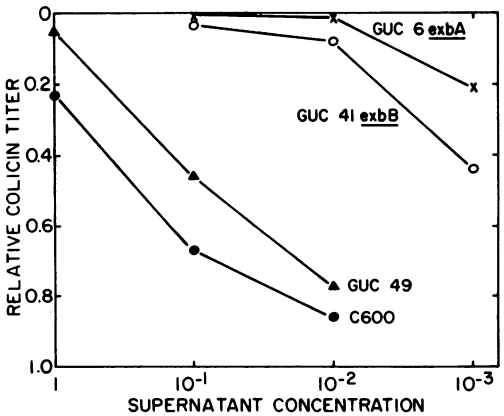

FIG. 1. *Production of colicin inhibitor by insensitive mutants. Supernatant fluids of overnight cultures of the indicated strains grown in M9 medium with amino acids plus 200 μM $FeCl_3$ were diluted in the same medium and assayed for colicin inhibitory content, plotted as relative colicin titer. Low colicin titers indicate high inhibitory power.*

TABLE 2. *Linkage of exbA and trp*[a]

Selected markers	No. tested	Unselected markers (%)						
		Thr⁺Leu⁺	Arg⁺	His⁺	Trp⁺	Met⁻	Pro⁻	Exb
Thr⁺Leu⁺	100		14	0	0	32	72	15
Arg⁺	100	45		0	0	88	43	30
His⁺	98	15	19		14	17	22	56
Trp⁺	98	14	8	16		5	16	88

[a] Strain GUW5 Hfr Cav *met pro exb-5 str*[s] was crossed with PA309 F⁻ *thr leu arg his trp str*[R]. All selective media contained 50 μg of streptomycin per ml.

To determine if *exb* mutants from C600 were *tonB* we used transduction of the *trpA* gene adjacent to *tonB*. Strain WA5028a, a double-point mutant in *trpA*, was infected with P1 grown on GUC12 *exbA*, GUC41*exbB*, or C600, and *trp*⁺ transductants were selected and tested for phage and colicin sensitivity (Table 3). Eighty-four percent of *trp*⁺ transductants from P1-GUC12 were *tonB* and *exb*. All transductants from C600 and GUC41 were wild type. Thus strain GUC12 *exb* is *tonA tonB*.

tonB mutants require supplementary iron for growth in minimal medium and are inhibited by chromium (16, 17). Growth of strain GUC12*exbA* was totally inhibited by $CrCl_3$ (Fig. 2) as were the *trpA*⁺ *tonB* transductants from this strain. Strain GUC12 grew poorly in minimal medium, but grew at wild-type rates if supplemented with 100 μM $FeCl_3$ (Fig. 3).

tonB mutants are exb. The above data indicate that certain mutants which we identify as *exbA* are *tonB*. To determine if this relationship is reciprocal, *tonB* mutants from other laboratories were tested. Strain VXII (provided by E. R. Signer) was prepared from X5050 by selection with colicin V and phage φ80*vir* and contains a deletion extending from *trp* to *lac* in prophage φ80d*lac*. Strain VXII was found to be colicin B insensitive and *exb*. Mu-induced *tonB* mutants (provided by M. Howe) were obtained from cells of strain M107 infected with phage mu by selection with colicin B and φ80*vir*. Phage mu was inserted in the *tonB* gene since *trpA*⁺ *tonB* derivatives by P1 transduction were lysogenic for mu. Mu-induced *tonB* as well as spontaneous *tonB* mutants from infected cells produced high levels of colicin inhibitor (Table 4).

tonB phenotype can be produced by insertion of lambda. Since *tonB* mutants frequently carry deletions, the various effects of the mutation might reflect a loss of different genes rather than pleiotropic effects of a lesion in a single gene. One test for pleiotropic effects was to isolate chromium-resistant revertants from an *exbA* strain and test for the other properties.

TABLE 3. *Co-transduction of exbA and trpA*

Donor	Trp⁺ transductants	Sensitivity to			*tonB* (%)
		Col B	φ80vir	T5	
C600	35	+	+	+	0
GUC12 *exbA*	27[a]	−	−	+	84
	5[b]	+	+	+	0
GUC41 *exbB*	50	+	+	+	0

[a] Six strains were tested and found to excrete inhibitor comparable to parent GUC12 *exbA*.

[b] Two strains were tested and found to excrete inhibitor comparable to recipient parent WA5028a.

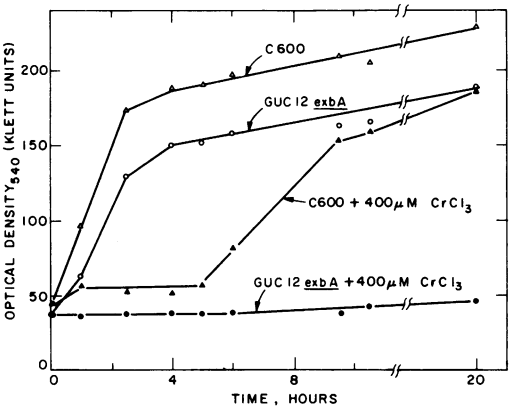

FIG. 2. *Chromium sensitivity of an exbA mutant. Chromium chloride was added to nutrient broth cultures in early log phase.*

Twelve such revertants from GUC12 *exbA* proved to be colicin insensitive, iron requiring, and *exb*, and yielded chromium-sensitive *tonB* strains by transduction of *trpA*⁺ to WA5028a. In these revertants, as possibly also in other *tonB* revertants selected for chromium resistance, the resistance may have arisen by extragenic suppression.

To test in another way if all the *tonB* effects can result from disfunction within a single cistron, lambda-induced *tonB* mutants were obtained in a strain of *E. coli* K-12 with a

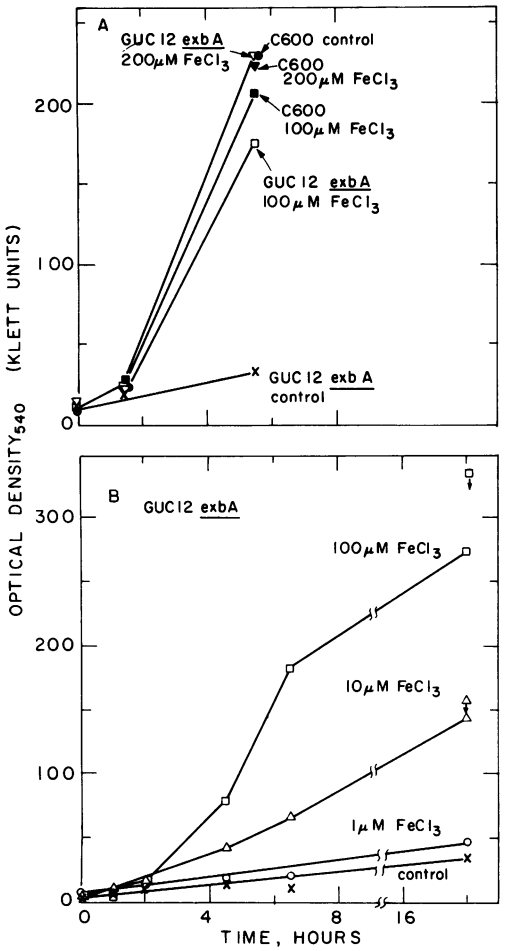

FIG. 3. *Iron requirement of an exbA mutant. A, Strains were grown in M9 medium supplemented with $FeCl_3$ as indicated. B, Strain GUC12, an exbA mutant from C600, was grown in M9 medium with 0, 1, 10, or 100 μM $FeCl_3$ added. Arrows indicate values corrected for pink color of the culture fluid.*

deletion of the normal attachment site. Shimada et al. (13) demonstrated that infection of a strain lacking the normal lambda attachment site yielded some lysogens in which the prophage had become inserted inside bacterial genes inactivating them. Reversion to wild type results in simultaneous loss of the prophage, indicating that insertion and excision of lambda occur with exact precision. Lambda-induced *tonB* mutants were obtained in strain B(583) Δ24(*gal bio λatt*) by infection with λ*cI*857 (temperature inducible) and selection with colicin B, φ80*vir* and λ*cI*60. Three temperature-sensitive phage-producing strains were obtained. High-titer lambda, prepared from strain 3LH9, mediated specialized *trp* transduction, indicating that the prophage was inserted next to the *trpA* gene. These lambda-induced *tonB* mutants were insensitive to colicins B, Ia, and Ib, required iron for growth in minimal medium, and were chromium sensitive and *exb* (Table 4). These phenotypes therefore can result from a mutation in a single cistron, assuming that the insertion of lambda does not result in polarity effects.

Genetic analysis of exbB mutants. Exb Met^- strains are T1 sensitive, chromium resistant, and grow on minimal medium without supplementary iron. The ExbB phenotype is probably due to a deletion since no Met^+ revertant colonies could be obtained by plating *exbB* cells on medium lacking methionine.

To locate the *exbB* gene on the *E. coli* chromosome, strain LD28 (an *exbB* mutant of Hfr strain P10 which inserts from min 79 in the order *arg, mtl, his*) was crossed with A2325 F^- *argE his mtl;* Arg^+ and Mtl^+ recombinants were selected at specific time intervals. The colicin-insensitive phenotype appeared between 30 and 40 min after mixing the strains, and was more closely linked to *mtl* than to *arg*. Correction for a lag in entry of markers located the *exbB* gene between 49 and 59 min. Conjugation of strain LD54 F^- *his exbB met* with Hfr KL16, which inserts from min 55 with his^+, an early marker, yielded his^+ recombinants that were *exbB* like the F^- parent. These data suggest that *exbB* is located between min 55 and 59.

Transduction of the *exbB* gene by phage P1 was performed to determine linkage to *serA* at min 57. Nine percent of $serA^+$ transductants (6 out of 66) with P1 grown on an *exbB* donor acquired colicin insensitivity and methionine

TABLE 4. *Production of colicin inhibitor by mu-induced or lambda-induced tonB mutants*

Supernatant fluid[a]	Inhibition of colicin (%)
tonB (mu)	96
ara-20 (mu)	16
tonB spontaneous	97
M107, parent	13
3LH4 *tonB* (lambda)	97
3LH5 *tonB* (lambda)	99
3LH9 *tonB* (lambda)	99
B(583)Δ24, parent	13

[a] Mu lysogens and controls were grown at 32 C in nutrient broth, and lambda lysogens and controls were grown at 30 C in M9 medium with 200 μM $FeCl_3$ and 1 μg of biotin per ml. Supernatant fluids were diluted 1:10 and titered for ability to inhibit colicin B.

auxotrophy. These transductants hyperexcreted colicin inhibitor in amounts comparable to the donor strain (Table 5). Thus, the *exbB* gene is located within about 1 min of *serA*.

The exbB phenotype is recessive. Strain LD63 *recA tsx exbB met serA* was mated with KL110/KLF16, which contains the F′ episome F116 including genes from *metC* at min 59 to *fuc* at min 54. Presumed merodiploid recombinants were selected on minimal medium supplemented only with methionine. These recombinants were sensitive to colicin B, produced 100 times less colicin inhibitor than the *exbB* parent, and approximately 3 times more than the wild type as shown in Table 6, and did not require methionine.

Characteristics of the methionine auxotrophy of exbB mutants. *exbB* strain LD28 required at least 10 μg of methionine per ml of minimal medium for optimal growth. The methionine requirement of strain GUC41 was compared to those of a *metA* and a *metE* mutant. GUC41 grew well with either homocysteine or cystathionine, suggesting that the auxotrophy is probably not due to a defect of conversion of cystathionine to methionine.

TABLE 5. *Co-transduction of exbB and serA*[a]

Supernatant fluid	Phenotype	Inhibition of colicin (%)
Transductant-1	Col^1Met^-	96
Transductant-2	Col^1Met^-	97
Transductant-3	Col^8Met^+	7
AB856 *serA* (recipient)	Col^8Met^+	0.5
LD28 *exbB* (donor)	Col^1Met^-	99

[a] Phage P1 grown on strain LD28 *exbB* was used to transduce $SerA^+$ to strain AB856 *serA*. Transductants were tested for colicin sensitivity (Col^1 or Col^8), for methionine requirement and for ability to excrete colicin inhibitor.

TABLE 6. *Production of colicin inhibitor by exbB/exbB⁺ merodiploids*[a]

Supernatant fluid	Inhibition of colicin (%)		
	Undiluted	1:10	1:100
Merodiploid-1	85	30	
Merodiploid-2	83	21	
Merodiploid-4	89	28	
LD63 *exbB* parent		97	84
KL110/KLF16 parent	62	5	

[a] Merodiploids were isolated from a mating of LD63 *serA exbB metL recA nalB tsx* with KL110/KLF16 F′ episome from min 54 to 59. Merodiploids were tested for production of colicin inhibitor in M9 medium with 200 μM $FeCl_3$ and 20 μg of methionine per ml.

Strains of *E. coli* and *S. typhimurium* known as *metK* mutants are characterized by loss of sensitivity to methionine analogs including ethionine and norleucine (9). The *exbB* mutant LD28 and its parent P10 were tested in minimal medium containing 5 mg of ethionine and 1 μg of methionine per ml, and were fully sensitive to ethionine (Fig. 4). Analogous experiments with norleucine were inconclusive since the sensitive control strain grew at all concentrations in the presence of methionine.

DISCUSSION

In this paper we have presented evidence concerning two types of mutations which have been mapped in different regions of the *E. coli* chromosome and cause excretion of large quantities of enterochelin in the presence of sufficient iron to repress synthesis of this compound in wild-type cells. *exbA* mutants excrete 1,000 times more enterochelin than wild-type strains, and the corresponding mutations are located in

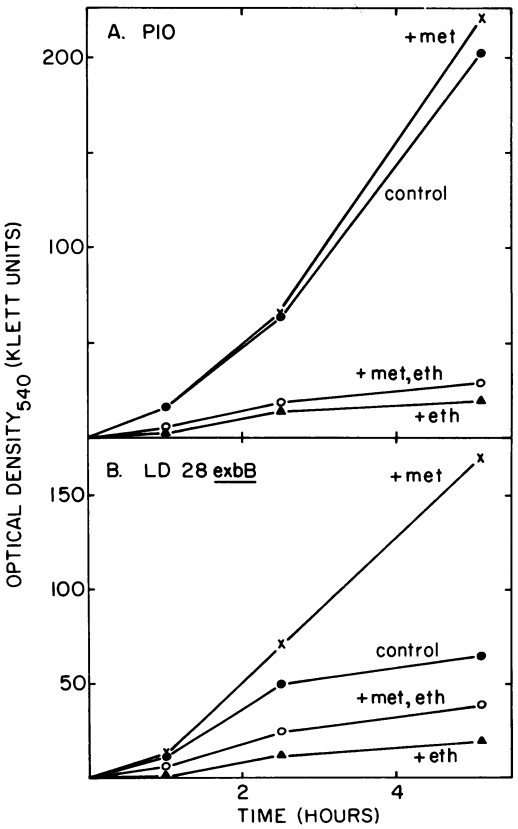

FIG. 4. *Ethionine sensitivity of an exbB mutant. Cultures were diluted into M9 medium with or without methionine (1 μg per ml) and ethionine (5 mg per ml). A, Parent strain P10. B, Strain LD28exbB.*

the *tonB* gene, and *tonB* mutants isolated independently are *exb*. The *exbB* mutations that cause a 250-fold increase in enterochelin production are located between min 56 and 58, may be mainly deletions, and are recessive with respect both to colicin sensitivity and enterochelin excretion. Neither *exb* trait could result from an operator constitutive mutation since these mutations are not linked to the *ent* genes for enterochelin synthesis, which map at min 14 (5, 10).

The *tonB* gene is involved in iron uptake, hence enterochelin excretion by *exbA* mutants may be related to the internal iron pools of these cells. Bryce and Brot (3) have calculated that synthesis of 2,3-dihydroxybenzolyserine occurs in cells when the intracellular iron level falls below 22×10^{-19} mol per cell. Assuming an *E. coli* cellular volume of 1 μm^3, the internal iron level necessary to repress synthesis is 2.2 mM. It is possible that *exbA* strains cannot accumulate enough iron to repress enterochelin biosynthesis and are therefore acting as constitutive. Excretion by *exbB* mutants, which are not defective in iron transport, may be due to failure to synthesize an iron-sensitive aporepressor of the enterochelin operon.

The methionine lesion of *exbB* mutants is satisfied by homocysteine and cystathionine. This observation and the map position rule out a mutation in known methionine biosynthetic enzymes or a defect in *metG* which maps near the histidine operon in *Salmonella typhimurium* (14) and probably at a comparable position in *E. coli* (1). Mutants insensitive to the methionine analog ethionine, known as *metK*, map at min 57 (11). The sensitivity of *exbB* strains to ethionine rules out the identity of *exbB* with *metK*. One possible basis of the *exbB* methionine auxotrophy is that these mutants lack a positive controlling substance for transcription or translation of one or more of the genes involved in methionine biosynthesis. The methionine auxotrophy associated with the mutation may be called *metL*.

The *tonB* region is one of the classical deletion systems in *E. coli* (4), and the exact relationship of fine structure to function has remained obscure. Our data on the lambda-containing *tonB* strains indicate that a defect in a single cistron can cause loss of all the properties attributed to the *tonB* gene, assuming no polarity effects from the inserted prophage. Gratia (6) found that different combinations of T1 and the colicin sensitivity mapped in this region and that these phenotypes are recessive. The recessive character indicates that an operator defect cannot be invoked to explain the pleiotropic effects of *tonB*. Further work is needed to clarify the relationship of the *tonB* gene product to the mutant phenotypes.

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