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Ethanolamine Utilization in *Salmonella typhimurium*

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Ethanolamine can serve as the sole source of carbon and nitrogen for *Salmonella typhimurium* if vitamin B_{12} is present to serve as a cofactor. The pathway for ethanolamine utilization has been investigated in order to understand its regulation and determine whether the pathway is important to the selective forces that have maintained the ability to synthesize B_{12} in *S. typhimurium*. We isolated mutants that are defective in ethanolamine utilization (*eut* mutants). These mutants defined a cluster of genes located between *purC* and *cysA* at 50 min on the *Salmonella* chromosome. A genetic map of the *eut* region was constructed. Included in the map are mutations which affect ethanolamine ammonia lyase, the first degradative enzyme, and mutations which affect the second enzyme in the pathway, acetaldehyde dehydrogenase. Transcriptional regulation of the *eut* genes was studied by using *eut-lac* operon fusions created by insertion of Mu d *lac*. Transcription is induced by the simultaneous presence of ethanolamine and B_{12} in the growth medium. The *eut* genes constitute a single unit of transcription. One class of mutations located at the promoter-distal end of the *eut* operon prevent induction of transcription.

Salmonella typhimurium can use ethanolamine as the sole source of carbon and nitrogen (13). Breakdown of ethanolamine requires the enzyme ethanolamine ammonia-lyase (EC 4.3.1.7). If provided with coenzyme B_{12}, this enzyme splits ethanolamine to acetaldehyde and ammonia (9, 37). Acetaldehyde generated by this reaction can be converted to acetylcoenzyme A by acetaldehyde dehydrogenase (EC 1.2.1.10) (26). Due to the B_{12} dependence of ethanolamine ammonia-lyase, *S. typhimurium* can use ethanolamine aerobically only if vitamin B_{12} is provided. Recently it was shown that *S. typhimurium* can synthesize B_{12} but only under anaerobic growth conditions (18, 24, 25). The endogenous B_{12} synthesis which occurs anaerobically is sufficient to permit use of ethanolamine as the sole nitrogen source without added B_{12}.

Ethanolamine ammonia-lyase is one of only two B_{12}-dependent enzymes known for *Escherichia coli* and *S. typhimurium*. The other B_{12}-dependent enzyme is a methyltransferase involved in the synthesis of methionine. The B_{12}-dependent transferase (*metH*) is not essential since, in the absence of B_{12}, an alternative transferase (*metE*) is capable of synthesizing methionine without B_{12} (19). Thus, neither of the B_{12}-dependent functions is essential under most growth conditions. Ethanolamine ammonia-lyase is only required if cells are growing on ethanolamine; the *metH* function is only required in mutants lacking the *metE* enzyme. However, some growth advantage may be provided by *metH* because the alternative (*metE*) enzyme is inefficient and represents several percent of total protein in cells growing without B_{12} (42). If no other B_{12}-dependent enzymes are discovered, one or both of these "nonessential" enzymes must contribute to the selective forces that have maintained the extensive array of genes required for synthesis and transport of vitamin B_{12}. Perhaps one or both of these B_{12}-dependent enzymes is of greater importance in nature than is immediately obvious.

Assimilation of ethanolamine may be an important part of the natural lifestyle of *S. typhimurium*. Phosphatidylethanolamine constitutes a large fraction of bacterial and animal phospholipids (34, 41). The enzymatic activities necessary to convert phosphatidylethanolamine to component fatty acids, glycerol, and ethanolamine have been identified in *E. coli* (28, 33). Therefore, the host diet, the bacteria present in the environment of *S. typhimurium*, and the epithelial cells of a host animal's intestinal tract could provide an abundant source of ethanolamine in nature. The benefits of ethanolamine assimilation may be a significant selective pressure for the maintenance of B_{12} biosynthesis.

Ethanolamine ammonia-lyase has been purified from *Clostridium* spp., and its mechanism of catalysis has been studied extensively (1). Ethanolamine ammonia-lyase from *E. coli* has been purified and characterized by Blackwell and Turner (3). The *E. coli* enzyme is very large (molecular weight, about 540,000) and is composed of subunits with molecular weights of 56,900 and 35,200 (3, 26). In *E. coli*, synthesis of ethanolamine ammonia-lyase is induced by the simultaneous presence of ethanolamine and B_{12}, a phenomenon termed concerted induction (4, 5). A coenzyme A-dependent acetaldehyde dehydrogenase activity is coordinately induced with ethanolamine ammonia-lyase activity (26). Mutants of *E. coli* have been described that are unable to use ethanolamine as a nitrogen source; these mutants lack ethanolamine ammonia-lyase activity in vitro. Mutants have also been described which synthesize both activities constitutively (4, 26, 27). Thus far, no detailed genetic study has been made of the system of ethanolamine utilization and the mechanism of its regulation.

The long-term goal of this study is to determine whether ethanolamine utilization is of sufficient physiological importance to account for the selective maintenance of the B_{12} biosynthetic capability. We also hope to understand the mechanism by which B_{12} and ethanolamine regulate ethanolamine ammonia-lyase synthesis. In this paper we describe the isolation and characterization of mutants defective in ethanolamine utilization. We show that the genes for ethanolamine utilization (*eut*) are located in a single operon mapping between *cysA* and *purC* at 50 min and that transcription of the *eut* operon is induced by the simultaneous presence of ethanolamine and B_{12}.

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TABLE 1. *S. typhimurium* strains used

Strain	Genotype[a]	Source
LT2	Wild type	Lab collection
TR255	*cysA1505 hisC777*	Lab collection
TR6583	*metE205 ara-9*	Lab collection
TR7015	*purC7*	Lab collection
TT220	*metE864*::Tn*10*	Lab collection
TT7610	*zeb-609*::Tn*10* *supD501*	Lab collection
TT8388	*zeb-609*::Tn*10* recA1/F'128 *zzf-1066*::Mu dA	Lab collection
TT10288	*hisD9953*::Mu dJ *hisA9944*::Mu dI	Lab collection
TT10508	*cysA1585*::Mu dA	Lab collection
TT10509	*cysA1586*::Mu dA	Lab collection
TT11567	*zfa-3644*::Tn*10d*Tet	This study
TT11568	*zfa-3645*::Tn*10d*Tet	This study
TT13438	*zfa-3646*::Tn*10*	This study
TT13440	*zfa-3648*::Tn*10*	This study
TT13744	*eut-162*::Mu dJ	This study
TT13749	*btu-1*::Mu dJ	This study
TT13767	*eut-189*::Mu dJ	This study
TT13775	*btu-2*::Mu dJ	This study
TT13894	*btu-3*::Tn*10*	This study

[a] Nomenclature for Mu d and Tn*10d*Tet strains is described in Materials and Methods.

MATERIALS AND METHODS

Bacterial strains and transposons. All stains used are derivatives of *S. typhimurium* LT2. Strains used in this study are described in Table 1. Some *eut* mutants are listed only in Fig. 3 by allele number and phenotype. We used two derivatives of the specialized transducing phage Mu dI(Amp^r *lac* cts) of Casadaban and Cohen (10). The first derivative, Mu dl-8, carries two mutations which make it conditionally defective for transposition (21). In this paper Mu dl-8 will be referred to as Mu dA. The second derivative, Mu dI1734, specifies Kan^r and lacks the Mu A and B genes necessary for transposition (11). In this paper Mu dI1734 will be referred to as Mu dJ.

Media and growth conditions. The complex medium was nutrient broth (0.8%; Difco Laboratories) with NaCl (0.5%). The minimal medium was E medium of Vogel and Bonner (39) with glucose (0.2%) as the carbon source. The carbon-free minimal medium was NCE (17), and the carbon- and nitrogen-free minimal medium was NCN (35). Ethanolamine hydrochloride (0.2%; Aldrich) was used as the carbon source in NCE medium, as the nitrogen source in NCN medium with glycerol (0.2%), or as both the carbon and nitrogen source in NCN medium. Glycerol (0.2%) or acetate (0.2%) was used as the carbon source in NCE medium. Cyanocobalamin (Sigma Chemical Co.) was used as the exogenous B_{12} source (100 μg/liter). Amino acids and purines were added to minimal media as required at the concentrations recommended previously (17). Cystine (0.058 mM) was added to nutrient broth used for growth of Cys auxotrophs. Antibiotics were used at the following concentrations in minimal and complex media, respectively: ampicillin, 15 or 30 μg/ml; kanamycin, 125 or 50 μg/ml; tetracycline, 10 or 20 μg/ml. Solid medium contained agar (1.5%; Difco) or, when a nitrogen source other than ammonia was used, Noble agar (1.5%; Difco). Cells were grown aerobically at 37°C unless indicated otherwise.

NCE medium containing glycerol and 25 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal; dissolved in *N,N*-dimethylformamide before addition) per ml was used in indicator plates for β-galactosidase activity.

The tetrazolium indicator medium of Bochner and Savageau (7) was modified and used with ethanolamine to identify mutants (Eut$^-$) unable to use ethanolamine. Ethanolamine indicator medium contained KH_2PO_4 (0.56%), K_2HPO_4 (0.24%), Bactopeptone (0.2%; Difco), $MgSO_4$ (1 mM), tetrazolium chloride (0.0025%), cyanocobalamin (0.1 μg/ml), ethanolamine hydrochloride (1%), and agar (1.5%). On ethanolamine indicator plates containing many colonies, Eut$^-$ strains form red colonies, while Eut$^+$ strains form white colonies.

Genetic techniques. All transductional crosses were performed with the high-frequency generalized transducing phage mutant P22 HT105/1 *int-201* (38). Most transductions were carried out by mixing 2×10^8 cells with 10^8 to 10^9 phage directly on the selective medium. When Kan^r transductants were selected, the cell culture and phage were incubated together nonselectively in liquid for 45 min before plating on the solid selective medium. Transductants were purified and made phage-free by streaking for single colonies on nonselective green indicator plates (12). Transductants were tested for phage sensitivity by cross-streaking against the clear-plaque mutant P22 H5.

In construction of the deletion map, many transductional crosses were done on a single plate with a nail block to apply 25 recipient strains to nutrient broth medium seeded with the donor P22 lysate. After 6 to 10 h of incubation, the plate was replica printed to NCN medium containing ethanolamine and B_{12} to select recombinants. The map was refined with higher-resolution crosses by using a whole plate for a single donor-recipient pair. Recombination between a point mutation and a deletion was scored as negative if two whole-plate crosses produced no *eut*$^+$ recombinants. Two such plates yield at least 2,000 *eut*$^+$ recombinants when the donor lysate is grown on a wild-type strain.

Hfrs were constructed as described by Chumley et al. (14) except that integration of the temperature-sensitive plasmid F'ts114 *lac zzf-20*::Tn*10* occurred by recombination between the plasmid *lac* sequences and the *lac* sequences present in *eut-18*::Mu dA. These recombinants were obtained by selecting for Tet^r at 42°C.

Chemical mutagenesis. Phage P22 lysates for localized mutagenesis experiments were concentrated and mutagenized with hydroxylamine by the method of Hong and Ames (20). Two donor strains were used, one containing a Tn*10* insertion adjacent to the promoter-proximal end of the *eut* region (TT13438) and the other containing a Tn*10* insertion adjacent to the promoter-distal end of the *eut* region (TT13440). These mutagenized lysates were used to transduce LT2 to Tet^r on ethanolamine indicator plates, and *eut* mutants were identified among the recombinants.

Diethyl sulfate (DES) mutagenesis of cell cultures was performed by incubating approximately 2×10^8 cells in 5 ml of E medium with 20 μl of DES for 20 to 60 min at 37°C. The cells were diluted 1,000-fold into E medium containing glucose, allowed to grow overnight, diluted again, and spread on complex medium to obtain single colonies. Eut$^-$ mutants were identified as those which did not grow on NCE medium containing ethanolamine and B_{12} but did grow on NCE medium containing acetate.

Tn*10* and Tn*10d*Tet mutagenesis. Tn*10* insertions were obtained by transducing pools of random Tn*10* insertions into the *eut-6*::Mu dA or *eut-18*::Mu dA fusion strain on Xgal indicator plates which contained tetracycline, ampicillin, ethanolamine, and B_{12} or on Xgal indicator plates which

contained only tetracycline and ampicillin. Tn*10* insertion mutants which formed lighter or darker blue colonies than the parent *eut*::Mu dA fusion strain were saved as potential *eut* mutants.

Tn*10* Del16 Del17 Tet^r (Tn*10d*Tet) is a small derivative of Tn*10* from which the transposase genes have been deleted (40). Tn*10d*Tet will transpose when introduced into strains containing the Tn*10* transposase gene on a multicopy plasmid. Tn*10d*Tet insertions were isolated from a pool of random Tn*10d*Tet insertions obtained from Tom Elliott.

Insertions of Tn*10* or Tn*10d*Tet adjacent to each end of the *eut* region were obtained by transducing the promoter-proximal *eut-18*::Mu dA mutant and the promoter-distal *eut-34*::Mu dA mutant to Tet^r with a P22 lysate grown on a pool of random Tn*10* or Tn*10d*Tet insertions. The Tet^r transductants were screened for those which had become Amp^s and Eut$^+$.

Deletion mutations in *eut* were isolated as tetracycline-sensitive derivatives of the Tn*10d*Tet strains TT11567 and TT11568; these were selected on complex medium by a modification (29) of the medium described by Bochner et al. (6). The selection and screening of tetracycline-sensitive derivatives was done at 40°C. Tetracycline-sensitive colonies which had become Eut$^-$ were identified by replica printing the colonies to minimal medium selective for tetracycline sensitivity prepared as described previously (6), except that ethanolamine and B_{12} were substituted for glucose.

Mu d mutagenesis. Random insertions of Mu dA were obtained by transducing the amber suppressor strain TT7610 (*supD501*) with phage P22 grown on the Mu dA insertion strain TT8388 on rich medium containing ampicillin. The Mu dA prophage in TT8388 cannot be inherited in the *Salmonella* chromosome by homologous recombination because the donor strain has Mu dA inserted in an *E. coli* F′ which contains no sequence homology with *Salmonella*. The Amp^r transductants were replica printed to NCE glycerol medium containing ampicillin and Xgal and to NCE glycerol medium containing ampicillin, Xgal, ethanolamine, and B_{12}. Each pair of replica plates was screened for colonies which were blue on one plate and white on the other. Mu dA insertion strains containing an amber suppressor mutation were grown at 30°C to prevent inactivation of the temperature-sensitive Mu repressor. To prevent further transposition of Mu dA, each insertion of interest was transduced into a suppressor-free background.

Mu dJ is defective for transposition. To isolate new Mu dJ insertion mutants, Mu dJ was transduced into a recipient on a transduced fragment that also included the Mu A and B genes of a Mu dI present near Mu dJ in the donor strain (23). Random insertions of Mu dJ were obtained by infecting LT2 with a P22 lysate grown on the Mu dI Mu dJ double lysogen TT10288 and selecting for Kan^r transductants. Strain TT10288 contains an insertion of Mu dJ in the *hisD* gene and an insertion of Mu dI in the *hisA* gene. P22 transducing particles which contain Mu dJ frequently contain the transposase genes of Mu dI as well, permitting Mu dJ to transpose (23). Insertions of Mu dI are not inherited along with Mu dJ because a complete Mu dI is too large to be packaged in a single P22 particle which also contains Mu dJ (22). Mutants unable to use ethanolamine were identified by replica printing the Kan^r transductants to NCE medium containing acetate and to NCE medium containing ethanolamine and B_{12}. Each Eut$^-$ insertion mutation was transduced into strain LT2 before characterization.

Directed deletion formation with Mu d prophages. Deletions generated by recombination between *eut* and *cysA* Mu dA insertions were isolated by three different crosses. The first cross, previously described by Hughes and Roth (22), is shown in Fig. 1A. The transduction is performed with a mixture of phage lysates made on two different Mu dA insertion strains. Recombination between the two Mu dA insertions can create a deletion or duplication of the material between the insertion sites (Fig. 1A). P22 lysates made on the Lac$^+$ *cysA1585*::Mu dA insertion strain TT10508 or the Lac$^-$ *cysA1586*::Mu dA insertion strain TT10509 were mixed with P22 lysates made on *eut*::Mu dA insertion strains and used to transduce LT2 to Amp^r. The transductants were replica printed to minimal medium and NCE medium containing ethanolamine, B_{12}, and cystine to identify deletion (*eut cys*) and duplication (*eut*$^+$ *cys*$^+$) strains.

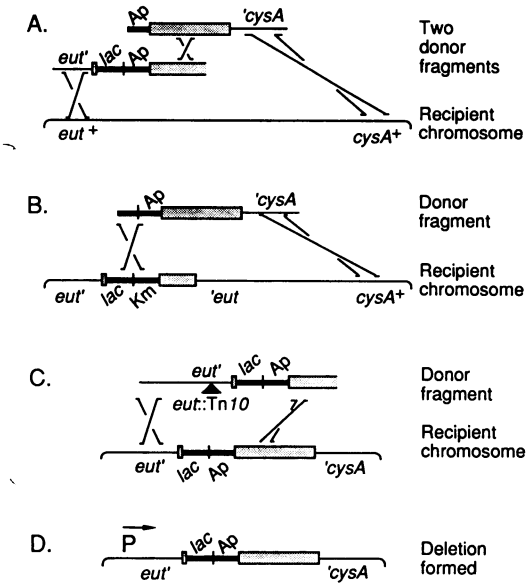

FIG. 1. Recombination between different Mu d insertions to generate deletions. The product of each of the three crosses (panels A, B, and C) is shown in panel D. (A) Recombination between an *eut*::Mu dA insertion on one transduced fragment and a *cysA*::Mu dA insertion on a second transduced fragment can generate a deletion of the sequences between the two insertions (22). This method was used to construct strains DEL-729 through DEL-753. (B) Recombination between a *cysA*::Mu dJ insertion on the transduced fragment and an *eut*::Mu dA insertion on the chromosome generates a deletion of the sequences between the two insertions. This method was used to construct DEL-754 through DEL-756. (C) Recombination between an *eut*::Mu dA insertion on the transduced fragment and a Mu dA insertion located at the join point of an *eut-cysA* deletion on the chromosome generates a new, shorter deletion. The *eut-208*::Tn*10* insertion remains adjacent to the new deletion and is removed in a subsequent transductional cross. This method was used to construct DEL-757 through DEL-763, DEL-863, and DEL-864.

Deletions ending at *eut*::Mu dJ insertions were also constructed in the cross shown in Fig. 1B. The *eut*::Mu dJ recipient strains were transduced to Amp^r with P22 made on the *cysA1585*::Mu dA strain or the *cysA1586*::Mu dA strain. The transductants were replica printed to minimal medium and NCE medium containing ethanolamine, B_{12}, and cystine to identify the deletion (Kan^s *eut*) strains. Deletions were formed between the two Mu d insertions in 5 to 10% of the transductants when the *eut*::Mu d and the *cysA*::Mu dA insertions were in the same orientation.

Additional deletions ending at *eut*::Mu d insertions were

constructed as shown in Fig. 1C. A strain containing a large Mu dA deletion (generated by either of the above methods) was transduced to Tet^r with P22 made on an *eut*::Tn*10* *eut*::Mu d double mutant. The *eut*::Tn*10* insertion could only be inherited by replacing the large deletion. Frequently this occurred by recombination between the two Mu d insertions, generating a shorter deletion with an endpoint at the donor Mu d insertion site. This technique has the advantage that all of the Tet^r transductants contain the expected new deletion. P22 lysates grown on the Tet^r colonies were used to transduce LT2 to Amp^r to obtain the new Mu dA deletion in a background without the *eut*::Tn*10* insertion.

Enzyme assays. Strains to be assayed for β-galactosidase activity were grown at 37°C to mid-log phase in NCE glycerol medium containing other additions as indicated in Table 3. β-Galactosidase activity was assayed in permeabilized cells as described previously (31). Strains to be assayed for ethanolamine ammonia-lyase activity and acetaldehyde dehydrogenase activity were grown at 37°C with vigorous gyratory shaking to an A_{650} of 0.6 in NCE glycerol medium containing other additions as indicated in Table 2. Cells were harvested by centrifugation, washed in 20 mM Tris hydrochloride (pH 7.6), suspended in 50 mM HEPPS (*N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid, pH 8)–200 mM KCl–1 mM dithiothreitol, and lysed by passage through a French pressure cell at 18,000 lb/in^2. The extract was first centrifuged at 24,000 × *g* for 45 min, and the supernatant was then centrifuged at 150,000 × *g* for 60 min. Portions of the second supernatant were frozen in dry ice-ethanol and stored at −70°C for up to 3 months before use. Ethanolamine ammonia-lyase was assayed by using a procedure similar to an assay described previously (8). Reaction mixtures (0.1 ml) contained 400 nmol of [2-^{14}C]ethanolamine hydrochloride (0.113 μCi/μmol; Amersham), 2 nmol of adenosylcobalamin (Sigma), 5 μmol of Tris hydrochloride (pH 7.5), 200 nmol of NADH, 10 U of yeast alcohol dehydrogenase (Sigma), and extract. The reactions were started by the addition of the adenosylcobalamin in dim red light and were incubated for 6 to 18 min at 37°C. The reactions were terminated by the addition of about 200 mg of Dowex 50W-X8 (hydrogen form) cation-exchange resin in 0.5 ml of 0.01 N HCl. The cation-exchange resin formed a complex with the unreacted ethanolamine, which was then removed by centrifugation. To determine the quantity of labeled acetaldehyde and ethanol formed, 0.2 ml of the supernatant was counted in a scintillation counter. Acetaldehyde dehydrogenase was assayed spectrophotometrically by following production of NADH at 340 nm as described previously (16). Cell protein was determined with the Bio-Rad protein assay with bovine serum albumin as the standard.

RESULTS

Mutants defective in ethanolamine utilization. Mutants unable to use ethanolamine as the sole source of nitrogen or carbon for growth fall into two phenotypic classes. Mutants unable to use ethanolamine as either the sole carbon or nitrogen source are designated Eut$^-$(N$^-$C$^-$). Mutants able to use ethanolamine as the sole nitrogen source but not as the sole carbon source are designated Eut$^-$(N$^+$C$^-$).

We identified insertions of Mu dA which caused *lacZ* to be induced by the presence of ethanolamine and B_{12} in the growth medium. Among 25,000 mutants with random Mu dA insertions screened, 41 formed blue colonies on Xgal indicator plates only when ethanolamine and B_{12} were present (mutations *eut-3* through *eut-43*). No mutants were identified which formed blue colonies only when ethanolamine and B_{12} were absent. All but two of the Mu dA insertions conferred the Eut$^-$(N$^-$C$^-$) phenotype. The two exceptional insertion strains (*eut-38* and *eut-41*) displayed no defect in growth on ethanolamine as a carbon or a nitrogen source. All of the *eut*::Mu dA insertions were linked by P22 transduction to the *eut-46*::Tn*10* insertion (described below) and therefore mapped at the same chromosomal locus.

Mu dJ insertions in *eut* were isolated by screening pools of random insertion mutants for mutants unable to grow on ethanolamine as the sole carbon source. This strategy was used to obtain Eut$^-$ Mu dJ insertions in both chromosomal orientations, in genes whose expression was not necessarily regulated by ethanolamine plus B_{12} and in genes whose expression was abolished by the insertion. Among 52 Mu dJ mutants that are unable to use ethanolamine as the carbon source, 48 showed linkage to *eut-46*::Tn*10* and thus carried mutations in the *eut* region. All 48 Mu dJ insertions linked to the *eut* locus conferred the Eut$^-$(N$^-$C$^-$) phenotype; 14 of these Mu dJ mutants showed induction of *lacZ* by ethanolamine plus B_{12}. Most of the unexpressed fusions were in the wrong orientation, but one, discussed later, appeared to affect a regulatory function. The four mutations unlinked to the *eut* operon will be discussed later.

Tn*10* insertions in *eut* were isolated as Lac$^-$ derivatives of *eut*::Mu dA fusion strains. This was done by looking for white colonies on Xgal indicator plates which contained ethanolamine and B_{12}. Several of the mutants were Lac$^-$ because of the polarity of their Tn*10* insertion on expression of the *lacZ* gene of a promoter-distal Mu dA insertion. Two strains became Lac$^-$ because the Tn*10* insertions disrupted genes whose activity is required for expression of the *eut* operon (discussed below). Several *eut*::Tn*10* insertions were isolated which apparently caused *lacZ* to be transcribed from a promoter located within the Tn*10* element (15). These insertions were identified as blue colonies on Xgal indicator plates lacking ethanolamine and B_{12}. All but one of the Tn*10* insertions isolated were linked by P22 transduction to the *eut-18*::Mu dA insertion; the unlinked Tn*10* insertion will be described later. The phenotype of each Tn*10* insertion in the absence of the *eut*::Mu dA insertion is indicated in Fig. 3; both Eut$^-$(N$^-$C$^-$) and Eut$^-$(N$^+$C$^-$) insertion strains were isolated.

A large number of hydroxylamine-induced point mutations in the *eut* region were isolated by localized mutagenesis as described in Materials and Methods. Several Eut$^-$ point mutants induced by DES were also isolated. These point mutations, which included both Eut$^-$(N$^-$C$^-$) and Eut$^-$(N$^+$C$^-$) types, are included in Fig. 3.

Deletions extending into the *eut* region were isolated for use in the construction of a deletion map. The deletions were obtained by applying positive selection for tetracycline sensitivity to strains containing Tn*10d*Tet inserted next to the *eut* region (on the *purC* side of *eut*; see below). Some mutants survived the selection because the Tn*10d*Tet element had been deleted. Tn*10d*Tet was used instead of Tn*10* to avoid the creation of deletions with the nonrandom endpoints characteristic of deletions induced by the Tn*10* transposase (32). We identified fifteen independent Eut$^-$ deletion strains. All of the deletions conferred the Eut$^-$(N$^-$C$^-$) phenotype because these deletions all remove the promoter end of the operon (see below).

A few Eut$^-$ mutants are defective in B_{12} transport. The mutant searches were done aerobically; under these conditions, *S. typhimurium* requires B_{12} for ethanolamine utiliza-

TABLE 2. Enzyme assays

Map region	Strain[a]	Supplements to minimal growth medium	Ethanolamine ammonia-lyase		Acetaldehyde dehydrogenase	
			Sp act[b]	% of control activity	Sp act[c]	% of control activity
None (control)	LT2	EA[d], B_{12}	45	(100)	1,825	(100)
	LT2	None	<0.5	<1	<15	<1
I–IV	*eut-237*	EA, B_{12}	<0.5	<1	<15	<1
I	*eut-64*	EA, B_{12}	14	31	580	32
	eut-101	EA, B_{12}	23	51	2,200	120
	eut-124	EA, B_{12}	18	40	1,375	75
	eut-91	EA, B_{12}	3	7	<15	<1
	eut-56	EA, B_{12}	18	40	<15	<1
	eut-55	EA, B_{12}	35	78	<15	<1
II	*eut-66*	EA, B_{12}	<0.5	<1	1,225	67
	eut-111	EA, B_{12}	18	40	1,500	82
	eut-114	EA, B_{12}	19	42	1,375	75
	eut-106	EA, B_{12}	2	4	1,550	85
	eut-107	EA, B_{12}	<0.5	<1	650	36
	eut-110	EA, B_{12}	<0.5	<1	630	35
III	*eut-100*	EA, B_{12}	<0.5	<1	<15	<1

[a] The strains are listed in their *eut* mutation map order from the promoter-proximal end to the promoter-distal end of the operon.
[b] Expressed as nanomoles of acetaldehyde and ethanol produced per minute per milligram of cell protein.
[c] Expressed as nanomoles of NADH produced per minute per milligram of cell protein.
[d] EA, Ethanolamine.

tion. Therefore, mutants defective in B_{12} transport would be expected among Eut$^-$ mutants. Mutants which are unable to transport B_{12} cannot use the B_{12}-dependent methyltransferase enzyme (*metH*), one of the two enzymes which can methylate homocysteine to give methionine (2). We checked the Eut$^-$ mutants for B_{12} transport function by eliminating the alternative B_{12}-independent methyltransferase enzyme (*metE*) and testing for growth on minimal medium supplemented only with B_{12}. All of the *metE* strains containing *eut*::Mu d and *eut*::Tn*10* insertions located in the *eut* region were able to grow on minimal medium supplemented with B_{12}. This indicates that mutations at the *eut* locus do not impair B_{12} transport.

Three Eut$^-$ mutations unlinked to the *eut* region did seem to prevent B_{12} uptake. The *metE* strains containing one of the unlinked Eut$^-$ Mu dJ insertions (*btu-1*::Mu dJ) or the unlinked Eut$^-$ Tn*10* insertion (*btu-3*::Tn10, isolated as a Lac$^-$ derivative of *eut-18*::Mu dA) were unable to grow on minimal medium containing the usual concentration of B_{12} (0.1 μg/ml). These strains exhibited full growth on minimal medium containing a high concentration of B_{12} (10 μg/ml), a phenotype characteristic of many B_{12} uptake mutants (2). These two insertions showed close linkage to each other (94% cotransduction by P22). The *metE* strain containing a third Eut$^-$ mutation (*btu-2*::Mu dJ, unlinked to the *eut* region) was able to grow on minimal medium containing the usual concentration of B_{12} but not on minimal medium containing a lower than usual B_{12} concentration (20 pg/ml) (C. Grabau, personal communication), suggesting that *btu-2*::Mu dJ is also defective in B_{12} uptake. The *metE* strains containing the two other Mu dJ insertions unlinked to the *eut* region (*eut-162* and *eut-189*) exhibited methionine-independent growth on minimal medium containing 2 pg of B_{12} per ml and were presumed to be normal for B_{12} transport. We have not investigated the basis for their Eut$^-$ phenotype.

Enzymatic defects in *eut* mutants. Extracts of several *eut* mutants were assayed for the two enzymes required for ethanolamine utilization, ethanolamine ammonia-lyase and acetaldehyde dehydrogenase (Table 2). As in *E. coli* (5), the enzyme activities were detected only in extracts of cells grown in the presence of ethanolamine and B_{12}. The genes for both ethanolamine ammonia-lyase and acetaldehyde dehydrogenase appeared to be located in the *eut* region. A strain containing a deletion of the entire *eut* region (*eut-237*) lacked both enzyme activities. Table 2 contains examples of *eut* point mutants which lacked one or both of the enzyme activities.

Location of the *eut* region. We located the *eut* region on the *S. typhimurium* map by using Hfr mapping crosses (14). The Hfr was formed by integrating F'ts114 *lac* into the *eut-18*::Mu dA insertion by recombination between the plasmid and Mu dA *lac* sequences as described previously (30). This Hfr transferred the *cysA* locus at a high frequency and transferred markers located further counterclockwise from *cysA* with gradually decreasing frequency (data not shown). These results indicate that the insertion is located between *cysA* at 50 map units and *guaA* at 52 map units on the chromosome (36).

The *eut* region was more precisely located with P22-mediated cotransduction crosses. The *eut* region was found to be located midway between *cysA* and *purC* at approximately 50.5 map units. A map of cotransduction frequencies between these markers is presented in Fig. 2. Using the formula described by Wu (43), we estimate from the cotransduction data that the *eut* region is between 12 and 15 kilobases long. While this is a rough estimate, it suggests that the operon is rather large.

Direction of transcription of *eut*. The *eut* operon was found to be transcribed in a counterclockwise orientation. The orientation of the *eut-18*::Mu d insertion was inferred from the Hfr mapping crosses described above. The gradient of chromosome transfer in these Hfr crosses indicated that the *lac* genes of *eut-18*::Mu dA are transcribed in the counterclockwise direction (30). The *lac* genes of *eut-18*::Mu dA are expressed from the *eut* promoter, indicating that transcription of the *eut* operon is also counterclockwise. The *eut* promoter is indicated in Fig. 2 on the *purC* side of the *eut* region.

Orientation of Mu d insertions and generation of deletions

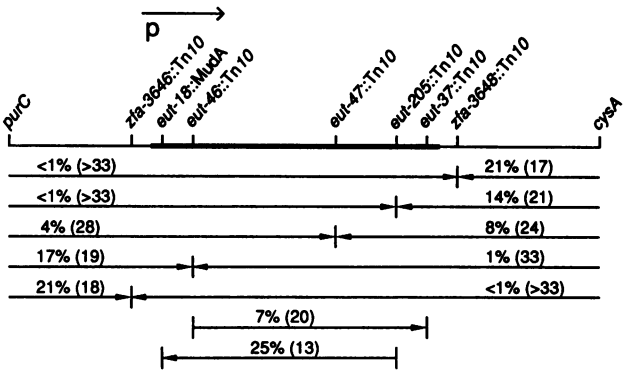

FIG. 2. Genetic linkage map of the *eut* region. P22 cotransduction frequencies are shown as percentages. The calculated distances (in kilobases) are shown in parentheses. The arrowheads point to the marker whose phenotype was scored in each cross. The location of the *eut* promoter (p) is indicated at the top.

by transductional crosses. Deletions can be generated by homologous recombination between Mu d insertions in *eut* and *cysA* only when the two Mu d insertions are in the same chromosomal orientation, as described in Materials and Methods and shown in Fig. 1. In these deletion strains, the sequences between the two parental Mu d insertions were deleted, leaving a single Mu d insertion at the deletion join point. Mu d deletions were constructed with 29 *eut*::Mu d insertions which showed induction of the *lac* genes by ethanolamine plus B_{12} and with eight *eut*::Mu d insertions which did not show induction. The inducible *eut*::Mu d insertions only formed deletions with the Lac$^+$ *cysA1585*::Mu dA insertion, while seven of the uninducible *eut*::Mu d insertions only formed deletions with the Lac$^-$ *cysA1586*::Mu dA insertion. This indicates that all of the inducible *eut*::Mu d insertions are in the same chromosomal orientation and that all but one of the uninducible *eut*::Mu d insertions are in the opposite orientation.

One unusual uninducible *eut*::Mu d insertion (*eut-156*::Mu dJ) formed a deletion to the *cysA1585*::Mu dA insertion and is therefore in the same orientation as the inducible *eut*::Mu d insertions. Evidence presented below suggests that this exceptional insertion disrupts a gene in the *eut* region required for induction of the *eut* promoter.

Genetic map of the *eut* region. The linear arrangements of 130 point and insertion mutation sites in *eut* and 50 deletions extending into *eut* are presented in Fig. 3. The data were obtained by transducing a series of deletion mutants with P22 lysates made on point or insertion mutants and scoring the frequencies of *eut*$^+$ recombinants produced. In addition,

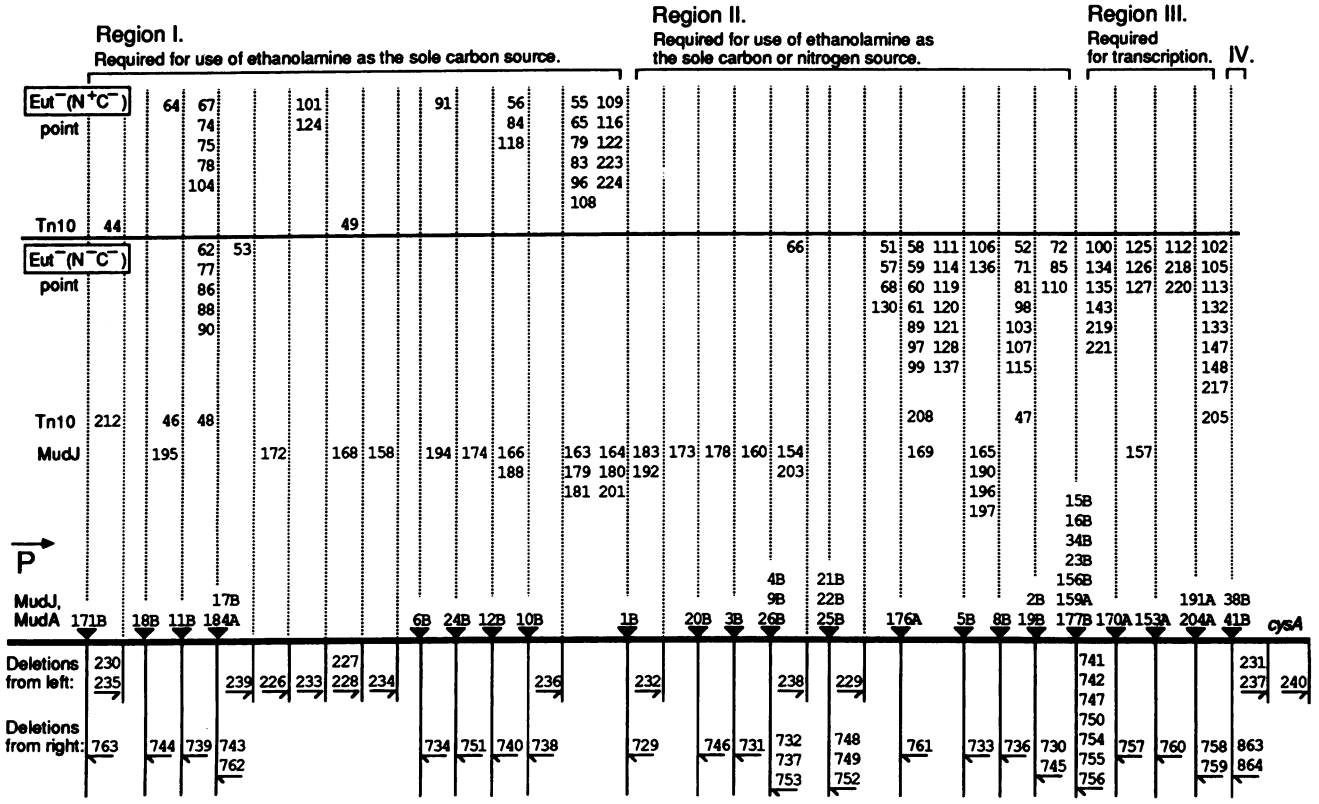

FIG. 3. Genetic map of the *eut* operon. The allele numbers of mutants which displayed the Eut$^-$(N$^+$C$^-$) phenotype are shown in the upper section, and the allele numbers of mutants which displayed the Eut$^-$(N$^-$C$^-$) phenotype are shown in the lower section. The heavy horizontal line represents the chromosome. All of the deletions used to construct the map originated outside the *eut* region. The deletion endpoints in *eut* are indicated with arrows, below the heavy horizontal line. Deletions *eut-226* through *eut-239* entered the operon from the left side. The *eut-cysA* deletions created by recombination between Mu d insertions entered the operon from the right side. These deletions are indicated by the DEL allele numbers 729 through 763, 863, and 864; the allele number of the *eut*::Mu d insertion used to generate a deletion is indicated above the corresponding DEL number. The chromosomal orientation of some Mu d insertions is indicated following the allele number; A indicates clockwise transcription of *lac*, and B indicates counterclockwise transcription of *lac* (22). Mu d insertions in the B orientation formed *eut-lac* operon fusions.

several intervals in the map were defined by crosses between two deletions. Four different regions, defined by mutant phenotypes, are indicated on the genetic map (Fig. 3).

The promoter-proximal third of the operon encodes activities necessary only for use of ethanolamine as the sole carbon source. All of the point mutations which conferred the Eut$^-$(N$^+$C$^-$) phenotype mapped in the promoter-proximal third of the map, region I (Fig. 3). The two Tn*10* insertions which conferred the Eut$^-$(N$^+$C$^-$) phenotype, *eut-44*::Tn*10* and *eut-49*::Tn*10*, also mapped in this region. These strains could grow on ethanolamine as a nitrogen source and complete the first step in ethanolamine utilization, catalyzed by ethanolamine ammonia-lyase. The enzyme which catalyzes the second step in ethanolamine utilization, acetaldehyde dehydrogenase, seems to be encoded in region I. Three of the Eut$^-$(N$^+$C$^-$) point mutants, *eut-55*, *eut-56*, and *eut-91*, lacked in vitro acetaldehyde dehydrogenase activity but retained in vitro ethanolamine ammonia-lyase activity (Table 2). In addition to acetaldehyde dehydrogenase, a second function must also be encoded in region I because the Eut$^-$(N$^+$C$^-$) point mutants *eut-64*, *eut-101*, and *eut-124* retained both enzyme activities in vitro. We have not yet determined the nature of this function. About one-quarter of the hydroxylamine-induced point mutations, three Tn*10* insertion mutations, and all of the Mu d insertion mutations mapping in region I conferred a Eut$^-$(N$^-$C$^-$) phenotype. These mutations are probably polar on downstream genes whose function is needed for growth on ethanolamine as a nitrogen source.

The promoter-distal half of the operon encodes activities required for use of ethanolamine as either a carbon or a nitrogen source. All mutations in the promoter-distal half of the map (regions II and III) conferred only the Eut$^-$(N$^-$C$^-$) phenotype. Two Eut$^-$(N$^-$C$^-$) mutants carrying point mutations in region II, *eut-107* and *eut-110*, lacked ethanolamine ammonia-lyase activity in vitro but retained much of the acetaldehyde dehydrogenase activity (Table 2). This suggests that the genes for ethanolamine ammonia-lyase are located in region II. Several other strains (*eut-91*, *eut-66*, and *eut-106*) had greatly reduced or eliminated ethanolamine ammonia-lyase activity in vitro as well. It is possible that the mutations in these strains exert a polar effect on transcription of a downstream gene(s) for ethanolamine ammonia-lyase. Complementation tests will be required to resolve these ambiguities. As in region I, an additional, undetermined function must also be encoded in region II because the Eut$^-$(N$^-$C$^-$) point mutants *eut-111* and *eut-114* retained both enzyme activities in vitro. The Eut$^-$(N$^-$C$^-$) strain containing the *eut-100* point mutation located at the far promoter-distal end of the operon (region III) lacked both enzyme activities in vitro. This strain did not seem to express the *eut* operon.

Some mutations in the promoter-distal part of the operon prevent transcription of *eut*. Several lines of evidence indicate that an activity encoded in the promoter-distal third of the operon (region III) is required for expression of *eut*. The *eut-205*::Tn*10* insertion (in region III) was isolated as a Lac$^-$ derivative of the *eut-18*::Mu dA fusion strain. Since this Tn*10* element mapped on the promoter-distal side of the affected Mu d insertion, we infer that the Tn*10* insertion cannot be exerting a polar effect and must be preventing initiation of transcription from the *eut* promoter. The unusual *eut-156*::Mu dJ insertion, which was properly orientated but did not show induction of *lac* in the presence of ethanolamine and B_{12}, also mapped in region III. In addition, expression of the *eut* operon was prevented by deletions which removed the right (promoter-distal) end of the operon. The strains carrying *eut-cys* deletions made with inducible *eut*::Mu d insertions retained the structural integrity of the *eut* promoter and the *lac* operon fusion but lost material distal to the fusion (Fig. 1). Induction of *lacZ* was examined by using Xgal indicator plates. In all but two of these deletion strains, *lacZ* was no longer inducible by ethanolamine plus B_{12} (the two exceptions are discussed below). Therefore, deletion of material between the *eut*::Mu d and the *cysA*::Mu d insertions appears to remove a function required for induction of the *eut* promoter.

To further define the extent of region III, we examined the effect of a series of point mutations on transcription of the *eut* operon. The promoter-proximal *eut-18*::Mu dA insertion was introduced into a series of point mutants by P22 transduction on Xgal indicator plates containing ampicillin, ethanolamine, and B_{12}. Point mutation *eut-100* and all point mutations located to the right of *eut-100* (Fig. 3) prevented expression of the *eut* operon, as judged by the failure of the double mutants to express the promoter-proximal *lac* fusion. Point mutations located between the *lac* fusion and *eut-100* (left of *eut-100*) had no effect on expression or regulation of *lac* expression. Therefore, the *eut-100* point mutation defines the border between regions II and III.

Since mutations in region III seemed to abolish a function required for expression of the *eut* genes, we hypothesize that this region is essential for activity of the operon's main promoter. If a protein is encoded in region III, its gene must be transcribed independently from the upstream genes in the operon, because Mu d insertions in the upstream genes did not appear to abolish the function of this region. Further evidence for an independent promoter is the fact that mutants carrying *eut-lac* fusions in region III retained a low level of expression in strains with a polar Tn*10* insertion in region I (data not shown).

The two exceptional *eut-cys* deletions that retained inducible expression of transcription were constructed by using the phenotypically Eut$^+$ Mu d insertions (*eut-38*::Mu dA and *eut-41*::Mu dA) as their left endpoint. The deletion strains remained Eut$^+$, and the *lac* fusions were still inducible by ethanolamine and B_{12}. This indicates that the Eut$^+$ insertions are located on the *cysA* side of all the genes required for ethanolamine utilization; deletions extending from the Eut$^+$ Mu d insertion sites to the right did not remove the region needed for induction of the *eut* promoter. The two Eut$^+$ Mu d insertions define region IV (Fig. 3).

All *eut*::*lac* fusions are in the same transcription unit. To determine whether all *eut*::Mu d fusions which showed induction of *lacZ* by ethanolamine plus B_{12} are located in the same transcription unit, we introduced a promoter-proximal Tn*10* insertion (*eut-46*::Tn*10*) into 41 *eut*::Mu dA insertion strains (*eut-3* through *eut-43*). Each of the double mutants was tested for transcriptional polarity of the Tn*10* insertion on the expression of *lacZ* by using Xgal indicator plates. The double mutants containing Mu d insertions located on the promoter-distal side of *eut-46*::Tn*10* displayed no induction of *lacZ* by ethanolamine plus B_{12}. A double mutant containing the *eut-46*::Tn*10* insertion and the single promoter-proximal Mu d insertion (*eut-18*::Mu dA) showed normal expression and regulation of the *lacZ* gene.

Transcriptional regulation of *eut*. We used the *eut*::*lac* operon fusions created by Mu d insertion to quantitatively examine transcriptional regulation in the *eut* region. In the fusion strains, changes in the level of β-galactosidase activity reflect changes in the level of *eut* transcription. The effect of ethanolamine and B_{12} on the level of β-galactosidase

TABLE 3. Regulation of β-galactosidase activity in *eut-lac* fusion strains

Insertion mutation[b]	Map region	β-Galactosidase sp act[a] of cells grown in medium with supplements:				Induction (fold) with EA + B_{12}
		None	EA[c]	B_{12}	EA + B_{12}	
eut-18::Mu dA	I	1.0	1.2	1.6	150	150
eut-22::Mu dA	II	2.2	2.2	2.6	102	46
eut-34::Mu dA[d]	II	3.0	2.8	3.4	44	15
eut-156::Mu dJ[d]	III	1.2	1.0	1.4	1.6	1.3
eut-38::Mu dA	IV	3.6	4.0	3.8	228	63

[a] Expressed as nanomoles of product produced per minute per A_{650} unit. Measured activity in Lac$^-$ strains was less than 0.2 U.
[b] Listed in their *eut* insertion mutation map order from the promoter-proximal end to the promoter-distal end of the operon.
[c] EA, Ethanolamine.
[d] These two insertion mutations mapped at the border of regions II and III; from the regulation patterns we infer that *eut-34*::Mu dA is in region II and *eut-156*::Mu dJ is in region III.

activity in five *lac* fusion strains is presented in Table 3. Induction of β-galactosidase activity requires the combination of ethanolamine plus B_{12} in all but one of the strains tested. The exceptional strain showed no induction of *lacZ* because it contained the *eut-156*::Mu dJ insertion in the promoter-distal region (region III) inferred to be essential for transcription induction.

DISCUSSION

We isolated a large collection of *S. typhimurium* mutants unable to use ethanolamine as a carbon source (*eut* mutants). Nearly all of the *eut* mutations mapped between *cysA* and *purC* at 50 min on the chromosome (Fig. 2). The *eut* mutations in this region fell into two phenotypic classes. Some mutants, designated Eut$^-$(N$^-$C$^-$), are unable to use ethanolamine as the sole carbon source or the sole nitrogen source. Other mutants, designated Eut$^-$(N$^+$C$^-$), are able to use ethanolamine as the sole nitrogen source but not as the sole carbon source.

The genetic map of the operon is divided into four regions, distinguished by the phenotype of mutations mapping in each region. All of the mutations which conferred the Eut$^-$(N$^+$C$^-$) phenotype are located in the promoter-proximal third of the *eut* operon, region I (Fig. 3). This region contains the gene for the second enzyme in ethanolamine utilization, acetaldehyde dehydrogenase. This gene was defined by several Eut$^-$(N$^+$C$^-$) mutants which lacked in vitro acetaldehyde dehydrogenase activity but retained in vitro ethanolamine ammonia-lyase activity (Table 2). Several Eut$^-$(N$^+$C$^-$) mutants carrying mutations in region I retained high levels of each activity in vitro, indicating that an undetermined function, required for use of ethanolamine as a carbon source, is also encoded in region I.

Region II, the central third of the operon, contains only mutations which conferred the Eut$^-$(N$^-$C$^-$) phenotype. The genes for ethanolamine ammonia-lyase are located in this region, defined by several mutants which lacked ethanolamine ammonia-lyase activity in vitro but retained in vitro acetaldehyde dehydrogenase activity. Several other region II mutants retained both in vitro activities, indicating that another undetermined function, required for use of ethanolamine as either a carbon or a nitrogen source, is encoded in region II.

Two *eut*::Mu d insertions mapping at the far distal end of the *eut* operon (region IV) had *lac* genes that were regulated in response to ethanolamine and B_{12} but caused no detectable defect in ethanolamine utilization or in *eut* transcription. These insertions may disrupt a gene whose function is related to ethanolamine utilization but which is not required for use of ethanolamine under the conditions tested, or they may be located between the last gene of the operon and the transcription terminator.

The genetic linkages suggest that the length of the operon is 12 to 15 kilobases. This implies that the operon could contain 10 to 15 genes, but characterization of mutants (and complementation data to be presented elsewhere) gives evidence for only six genes necessary for ethanolamine utilization. The discrepancy suggests that additional genes are present in the operon whose loss does not lead to the Eut$^-$ phenotype. An alternative and likely explanation is that the transductional crosses overestimated distances. For example, if a favored P22 packaging site exists within the operon, fewer transductants would inherit markers at both ends of the operon.

Transcription of the *eut* genes required the simultaneous presence of ethanolamine and B_{12}. While it is unusual that induction of an operon requires a cofactor of one of the enzymes in that operon, B_{12} is an unusual cofactor. The cell only makes B_{12} anaerobically; under aerobic conditions the availability of B_{12} depends on its presence in the medium. Thus, B_{12} is a cofactor whose presence depends on growth conditions; it seems appropriate that its presence might be a prerequisite for gene expression.

Strains containing mutations in region III displayed the Eut$^-$(N$^-$C$^-$) phenotype and lacked both in vitro activities. These mutations prevented induction of transcription of the *eut* genes by ethanolamine and B_{12}. Region III could encode genes required to transport the ethanolamine necessary for induction, or it could encode a positive regulator of *eut* transcription. Work currently in progress indicates that region III is not necessary for use of ethanolamine as a nitrogen source when the *eut* genes are transcribed from a plasmid promoter (D. Roof and J. Roth, unpublished results). This result suggests that region III is not directly required for ethanolamine utilization and is more likely to encode a positive regulatory element. It appears that region III is transcribed from a weak promoter which is independent of the promoter for the upstream genes, because polar insertion mutations in the upstream genes did not completely abolish the function of region III. Mu d *lac* operon fusions downstream of region III (in region IV) appeared to be transcribed from the primary promoter as well as the weak promoter for region III. This suggests that region III is transcribed from both the primary promoter and a weak internal promoter.

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