Ethanalamine Utilization in *Salmonella typhimurium*

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Ethanalamine can serve as the sole source of carbon and nitrogen for *Salmonella typhimurium* if vitamin B₁₂ is present to serve as a cofactor. The pathway for ethanalamine 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 B₁₂ in *S. typhimurium*. We isolated mutants that are defective in ethanalamine 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 ethanalamine 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 ethanalamine and B₁₂ 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 ethanalamine as the sole source of carbon and nitrogen (13). Breakdown of ethanalamine requires the enzyme ethanalamine ammonia-lyase (EC 4.3.1.7). If provided with coenzyme B₁₂, this enzyme splits ethanalamine 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 B₁₂ dependence of ethanalamine ammonia-lyase, *S. typhimurium* can use ethanalamine aerobically only if vitamin B₁₂ is provided. Recently it was shown that *S. typhimurium* can synthesize B₁₂ but only under anaerobic growth conditions (18, 24, 25). The endogenous B₁₂ synthesis which occurs anaerobically is sufficient to permit use of ethanalamine as the sole nitrogen source without added B₁₂.

Ethanalamine ammonia-lyase is one of only two B₁₂-dependent enzymes known for *Escherichia coli* and *S. typhimurium*. The other B₁₂-dependent enzyme is a methyltransferase involved in the synthesis of methionine. The B₁₂-dependent transferase (*metH*) is not essential since, in the absence of B₁₂, an alternative transferase (*metE*) is capable of synthesizing methionine without B₁₂ (19). Thus, neither of the B₁₂-dependent functions is essential under most growth conditions. Ethanalamine ammonia-lyase is only required if cells are growing on ethanalamine; 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 B₁₂ (42). If no other B₁₂-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 B₁₂. Perhaps one or both of these B₁₂-dependent enzymes is of greater importance in nature than is immediately obvious.

Assimilation of ethanalamine 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 ethanalamine 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 ethanalamine in nature. The benefits of ethanalamine assimilation may be a significant selective pressure for the maintenance of B₁₂ biosynthesis.

Ethanalamine ammonia-lyase has been purified from *Clostridium* spp., and its mechanism of catalysis has been studied extensively (1). Ethanalamine 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 ethanalamine ammonia-lyase is induced by the simultaneous presence of ethanalamine and B₁₂, a phenomenon termed concerted induction (4, 5). A coenzyme A-dependent acetaldehyde dehydrogenase activity is coordinately induced with ethanalamine ammonia-lyase activity (26). Mutants of *E. coli* have been described that are unable to use ethanalamine as a nitrogen source; these mutants lack ethanalamine 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 ethanalamine utilization and the mechanism of its regulation.

The long-term goal of this study is to determine whether ethanalamine utilization is of sufficient physiological importance to account for the selective maintenance of the B₁₂ biosynthetic capability. We also hope to understand the mechanism by which B₁₂ and ethanalamine regulate ethanalamine ammonia-lyase synthesis. In this paper we describe the isolation and characterization of mutants defective in ethanalamine utilization. We show that the genes for ethanalamine 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 ethanalamine and B₁₂.

* Corresponding author.
TABLE 1. S. typhimurium strains used

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* Nomenclature for Mu d and Tn10dTet strains is described in Materials and Methods.

MATERIALS AND METHODS

Bacterial strains and transposons. All strains 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 dl(Amp’, 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 dl1734, specifies Kan’ and lacks the Mu A and B genes necessary for transposition (11). In this paper Mu dl1734 will be referred to as Mu dl.

Media and growth conditions. The complex medium was nutrient broth (0.8%; Difco Laboratories) and 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 NCM (35). Ethanolamine hydrochloride (0.2%; Aldrich) was used as the carbon source in NCE medium, as the nitrogen source in NCM medium with glyceral (0.2%), or as both the carbon and nitrogen source in NCM 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 B₁₂ 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-

J. Bacteriol.
contained only tetracycline and ampicillin. Tn10 insertion mutants which formed lighter or darker blue colonies than the parent eut::Mu dA fusion strain were saved as potential eut mutants.

Tn10 Del16 Del17 Tet' (Tn10dTet) is a small derivative of Tn10 from which the transposase genes have been deleted (40). Tn10dTet will transpose when introduced into strains containing the Tn10 transposase gene on a multicopy plasmid. Tn10dTet insertions were isolated from a pool of random Tn10dTet insertions obtained from Tom Elliott.

Insertions of Tn10 or Tn10dTet 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' with a P22 lysate grown on a pool of random Tn10 or Tn10dTet insertions. The Tet' transductants were screened for those which had become Amp' and Eut'.

Deletion mutations in eut were isolated as tetracycline-sensitive derivatives of the Tn10dTet 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 B12 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' transductants were replica printed to NCE glycerol medium containing ampicillin and Xgal and to NCE glycerol medium containing ampicillin, Xgal, ethanolamine, and B12. 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 dJ 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 dJ Mu dJ double lysogen TT10288 and selecting for Kan' transductants. Strain TT10288 contains an insertion of Mu dJ in the hisD gene and an insertion of Mu dJ in the hisA gene. P22 transducing particles which contain Mu dJ frequently contain the transposase genes of Mu dJ as well, permitting Mu dJ to transpose (23). Insertions of Mu dJ are not inherited along with Mu dJ because a complete Mu dJ 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' transductants to NCE medium containing acetate and to NCE medium containing ethanolamine and B12. Each Eut' insertion mutation was transduced into strain LT2 before characterization.

**Directed deletion formation with Mu d prophages.** Dele-

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**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::Tn10 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-964. insertions 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'. The transductants were replica printed to minimal medium and NCE medium containing ethanolamine, B12, and cystine to identify deletion (eut cys) and duplication (eut' cys') strains.

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' 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, B12, and cystine to identify the deletion (Kan" 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' with P22 made on an eut::Tn10 eut::Mu d double mutant. The eut::Tn10 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' transductants contain the expected new deletion. P22 lysates grown on the Tet' colonies were used to transduce LT2 to Amp' to obtain the new Mu dA deletion in a background without the eut::Tn10 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 gyrate shaking to an A650 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 KC1–1 mM dithiothreitol, and lysed by passage through a French pressure cell at 18,000 lb/in². 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 [2-3H]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 μl 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 B12 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 B12 were present (mutations eut-3 through eut-43). No mutants were identified which formed blue colonies only when ethanolamine and B12 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::Tn10 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 B12 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::Tn10 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 B12. 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.

Tn10 insertions in eut were isolated as Lac− derivatives of Mu dA fusion strains. This was done by looking for white colonies on Xgal indicator plates which contained ethanolamine and B12. Several of the mutants were Lac− because of the polarity of their Tn10 insertion on expression of the lacZ gene of a promoter-distal Mu dA insertion. Two strains became Lac− because the Tn10 insertions disrupted genes whose activity is required for expression of the eut operon (discussed below). Several eut::Tn10 insertions were isolated which apparently caused lacZ to be transcribed from a promoter located within the Tn10 element (15). These insertions were identified as blue colonies on Xgal indicator plates lacking ethanolamine and B12. All but one of the Tn10 insertions were linked by P22 transduction to the eut-18::Mu dA insertion; the unlinked Tn10 insertion will be described later. The phenotype of each Tn10 insertion in the absence of the eut::Mu dA insertions is indicated in Fig. 3; both Eut− (N−C−) and Eut− (N+C−) insertion strains were isolated.

A large number of hydroxyamine-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 Tn10dTet inserted next to the eut region (on the purC side of eut; see below). Some mutants survived the selection because the Tn10dTet element had been deleted. Tn10dTet was used instead of Tn10 to avoid the creation of deletions with the nonrandom endpoints characteristic of deletions induced by the Tn10 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 B12 transport. The mutant searches were done aerobically; under these conditions, S. typhimurium requires B12 for ethanolamine utiliza-
tion. Therefore, mutants defective in B₁₂ transport would be expected among Eut⁻ mutants. Mutants which are unable to transport B₁₂ cannot use the B₁₂-dependent methyltransferase enzyme (metH), one of the two enzymes which can methylate homocysteine to give methionine (2). We checked the Eut⁻ mutants for B₁₂ transport function by eliminating the alternative B₁₂-independent methyltransferase enzyme (metE) and testing for growth on minimal medium supplemented only with B₁₂. All of the metE strains containing eut::Mu d and eut::Tn10 insertions located in the eut region were able to grow on minimal medium supplemented with B₁₂. This indicates that mutations at the eut locus do not impair B₁₂ transport.

Three Eut⁻ mutations unlinked to the eut region did seem to prevent B₁₂ uptake. The metE strains containing one of the unlinked Eut⁻ Mu d insertions (btu-1::Mu d) or the unlinked Eut⁻ Tn10 insertion (btu-3::Tn10, isolated as a Lac⁺ derivative of eut-18::Mu d) were unable to grow on minimal medium containing the usual concentration of B₁₂ (0.1 µg/ml). These strains exhibited full growth on minimal medium containing a high concentration of B₁₂ (10 µg/ml), a phenotype characteristic of many B₁₂ 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 d), which was able to grow on minimal medium containing the usual concentration of B₁₂ but not on minimal medium containing a lower than usual B₁₂ concentration (20 pg/ml) (C. Grabau, personal communication), suggesting that btu-2::Mu d is also defective in B₁₂ uptake. The metE strains containing the two other Mu d insertions unlinked to the eut region (eut-162 and eut-189) exhibited methionine-independent growth on minimal medium containing 2 pg of B₁₂ per ml and were presumed to be normal for B₁₂ 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 ethanamine utilization, ethanamine 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 ethanamine and B₁₂. The genes for both ethanamine 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's t114 lac into the eut-18::Mu d lac sequence, 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 and guaA at 50 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 that 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 d are transferred in the counterclockwise direction (30). The lac genes of eut-18::Mu d 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**

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**TABLE 2. Enzyme assays**

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<td>&lt;0.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>eut-111</td>
<td>EA⁺, B₁₂</td>
<td>18</td>
<td>40</td>
<td>1,500</td>
</tr>
<tr>
<td>eut-114</td>
<td>EA⁺, B₁₂</td>
<td>19</td>
<td>42</td>
<td>1,375</td>
</tr>
<tr>
<td>eut-106</td>
<td>EA⁺, B₁₂</td>
<td>2</td>
<td>4</td>
<td>1,550</td>
</tr>
<tr>
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<td>EA⁺, B₁₂</td>
<td>&lt;0.5</td>
<td>&lt;1</td>
<td>650</td>
</tr>
<tr>
<td>eut-110</td>
<td>EA⁺, B₁₂</td>
<td>&lt;0.5</td>
<td>&lt;1</td>
<td>630</td>
</tr>
<tr>
<td>III</td>
<td>eut-100</td>
<td>EA⁺, B₁₂</td>
<td>&lt;0.5</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* The strains are listed in their eut mutation map order from the promoter-proximal end to the promoter-distal end of the operon.

° Expressed as nanomoles of acetaldehyde and ethanol produced per minute per milligram of cell protein.

**| EA, Ethanolamine.**
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 B12 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,

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

![Diagram](image)

**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.
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 Tn10 insertions which conferred the Eut⁺ (N⁺C⁻) phenotype, eut-44:TN10 and eut-49::TN10, also mapped in this region. These strains could grow on ethanolamine as a nitrogen source and complemented 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 Tn10 insertion mutations, and all of the Mu d insertion mutations mapping in region I conferred an 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:TN10 insertion (in region III) was isolated as a Lac⁻ derivative of the eut-18::Mu dA fusion strain. Since this Tn10 element mapped on the promoter-distal side of the affected Mu d insertion, we infer that the Tn10 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 oriented but did not show induction of lac in the presence of ethanolamine and B12, 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 B12 (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 B12. 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 lower level of expression in strains with a polar Tn10 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 retained Eut⁺, and the lac fusions were still inducible by ethanolamine and B12. The Eut⁺ mutants, eut-40 and eut-43, are candidates for the promoter-distal end of the operon. 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 B12 are located in the same transcription unit, we introduced a promoter-proximal Tn10 insertion (eut-46::TN10) into 41 eut::Mu dA insertion strains (eut-3 through eut-43). Each of the double mutants was tested for transcriptional polarity of the Tn10 insertion on the expression of lacZ by using Xgal indicator plates. The double mutants containing Mu d insertions located on the promoter-distal end of eut-46::TN10 displayed no induction of lacZ expression by ethanolamine plus B12. A double mutant containing the eut-46::TN10 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 B12 on the level of β-galactosidase
activity in five lac fusion strains is presented in Table 3. Induction of β-galactosidase activity requires the combination of ethanolamine plus B12 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 strain (*N-C-*), are unable to use ethanolamine as the sole carbon source or the sole nitrogen source. Other mutants, designated Eut strain (*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 to each region. All of the mutations which conferred the Eut strain (*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 strain (*N- C- *) mutants which lacked in vitro acetaldehyde dehydrogenase activity but retained in vitro ethanolamine-ammonia-lyase activity (Table 2). Several Eut strain (*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 strain (*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 B12 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 strain 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 B12. While it is unusual that induction of an operon requires a cofactor of one of the enzymes in that operon, B12 is an unusual cofactor. The cell only makes B12 anaerobically; under aerobic conditions the availability of B12 depends on its presence in the medium. Thus, B12 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 strain (*N-C-*) phenotype and lacked both in vitro activities. These mutations prevented induction of transcription of the *eut* genes by ethanolamine and B12. 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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**Table 3. Regulation of β-galactosidase activity in *eut-lac* fusion strains**

<table>
<thead>
<tr>
<th>Insertion mutation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Map region</th>
<th>β-Galactosidase sp act&lt;sup&gt;b&lt;/sup&gt; of cells grown in medium with supplements:</th>
<th>Induction (fold) with</th>
<th>EA + B12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>EA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B12</td>
</tr>
<tr>
<td><em>eut-18::Mu dA</em></td>
<td>I</td>
<td>1.0</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td><em>eut-22::Mu dA</em></td>
<td>II</td>
<td>2.2</td>
<td>2.2</td>
<td>2.6</td>
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<tr>
<td><em>eut-34::Mu dA</em></td>
<td>II</td>
<td>3.0</td>
<td>2.8</td>
<td>3.4</td>
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<tr>
<td><em>eut-156::Mu dJ</em></td>
<td>III</td>
<td>1.2</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td><em>eut-38::Mu dA</em></td>
<td>IV</td>
<td>3.6</td>
<td>4.0</td>
<td>3.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as nanomoles of product produced per minute per A<sub>600</sub> unit. Measured activity in Lac<sup>-</sup> strains was less than 0.2 U.

<sup>b</sup> Listed in their order of mapped order (region IV) from the promoter-proximal end to the promoter-distal end of the operon.

<sup>c</sup> EA, Ethanolamine.

<sup>d</sup> 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.
ACKNOWLEDGMENTS

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LITERATURE CITED