Functions Required for Vitamin B₁₂-Dependent Ethanolamine Utilization in *Salmonella typhimurium*

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When B₁₂ is available, *Salmonella typhimurium* can degrade ethanolamine to provide a source of carbon and nitrogen. B₁₂ is essential since it is a cofactor for ethanolamine ammonia-lyase, the first enzyme in ethanolamine breakdown. *S. typhimurium* makes B₁₂ only under anaerobic conditions; in the presence of oxygen, exogenous B₁₂ must be provided to permit ethanolamine utilization. Genes required for ethanolamine utilization are encoded in the *eut* operon. For complementation testing, an F' plasmid containing the *eut* genes was constructed by transposition of the *eut* operon (flanked by two Tn10 elements) to an existing F' plasmid. Complementation tests defined six genes in the *eut* operon. Three of these genes encode enzymes known to be involved in degradation of ethanolamine: ethanolamine ammonia-lyase (*eutB* and *eutC*) and acetaldehyde dehydrogenase (*eutE*). One gene (*eutR*) seems to encode a positive regulatory protein required for induction of transcription of *eut*. The function of one of the remaining two genes (*eutA*) was shown to be required for ethanolamine utilization only when cyano-B₁₂ or hydroxy-B₁₂ were the precursors of the adenosyl-B₁₂ cofactor of ethanolamine ammonia-lyase; *eutA* mutants could use ethanolamine as the nitrogen source only when adenosyl-B₁₂ was provided. No function has been assigned to the *eutD* gene, which is required for use of ethanolamine as a carbon source. Ethanolamine uptake assays of *eut* mutants suggest that no ethanolamine permease is encoded in the *eut* operon.

Ethanolamine can be cleaved to acetaldehyde and ammonia by the enzyme ethanolamine ammonia-lyase when adenosylcobalamin (Ado-B₁₂) is available to serve as a cofactor (4, 28). This enzyme permits *Salmonella typhimurium* to use ethanolamine as the sole source of carbon and nitrogen (9). *S. typhimurium* can synthesize B₁₂ de novo but only under anaerobic growth conditions (Fig. 1) (23). The ability to synthesize B₁₂ requires an extensive array of genes, but the metabolic importance of B₁₂ and the reason B₁₂ synthesis occurs only during anaerobic growth are not understood (15, 22). In addition to ethanolamine ammonia-lyase, three other *S. typhimurium* enzymes are known to require a B₁₂ cofactor. Propanediol dehydratase (dl-1,2-propanediol hydrolyase; EC 4.2.1.28) enables propanediol to be used as the sole carbon source (30) and has only recently been detected in *S. typhimurium* (R. Jeter, personal communication). A B₁₂-dependent methyl transferase (encoded by the *metH* gene) methylates homocysteine to form methionine (35). The *metH* enzyme is not essential for methionine biosynthesis because a B₁₂-independent enzyme (encoded by the *metE* gene) can catalyze the same reaction (16). Vitamin B₁₂ is also required for formation of the modified base queuosine present in the anticonodon loop of some tRNAs, but this modification is not essential under standard growth conditions (18). The genetics of ethanolamine utilization is being studied as a tool for analysis of the B₁₂ biosynthetic pathway, to determine how genes are regulated in response to B₁₂ availability, and to learn the physiological importance of B₁₂ to *S. typhimurium*.

During aerobic growth, B₁₂ must be obtained from the growth medium to permit ethanolamine utilization. The form of B₁₂ encountered in nature is probably hydroxy-B₁₂ (HO-B₁₂). While cyano-B₁₂ (CN-B₁₂; commercial vitamin B₁₂) is generally provided in laboratory media, neither form of B₁₂ can be used directly as the cofactor for ethanolamine ammonia-lyase. To form Ado-B₁₂ from exogenous B₁₂, the cell must transport the B₁₂, reduce the central cobalt atom, and add an adenosyl group (Fig. 1) (20). A high-affinity B₁₂ transport system has been described for *Escherichia coli* (13); a similar system appears to exist in *S. typhimurium*. The enzymes responsible for reduction and adenosylation have been studied in several species of bacteria (5, 31, 33). Mutants of *E. coli* defective in the *btuR* gene have a decreased pool of Ado-B₁₂, but still contain enough Ado-B₁₂ to permit growth on ethanolamine (25). J. Escalante-Semerena, S.-J. Suh, and J. Roth (unpublished results) have isolated mutants (*cobA*) in the same region of the *S. typhimurium* chromosome as *btuR* of *E. coli*. The *cobA* mutants are completely defective in ethanolamine utilization (using CN-B₁₂) when grown in glucose medium with heavy aeration; under these growth conditions, *cobA* mutants can use ethanolamine only if Ado-B₁₂ is provided. Anaerobically, the *cobA* mutants express a gene in the CobI operon that can perform the missing (*cobA*) function, permitting adenosylation of assimilated CN-B₁₂ and HO-B₁₂. Apparently, at least one of the steps in Ado-B₁₂ formation can be catalyzed by either one of two separately encoded enzymes. Since ethanolamine ammonia-lyase requires Ado-B₁₂ and the B₁₂-dependent methyl transferase does not, one would expect mutants defective in adenosylation to be defective in ethanolamine degradation but to be capable of using B₁₂ for methionine synthesis. One might expect that functions involved in adenosylation of B₁₂ would be encoded in the same operon as the other functions required for ethanolamine utilization.

Mutants defective in ethanolamine utilization define the *eut* operon, located at about 50 min on the *S. typhimurium* chromosome (27). Induction of transcription of the *eut* operon requires the simultaneous presence of ethanolamine and B₁₂, but the mechanism by which this regulation pattern

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is achieved is not fully understood. A genetic map of eut includes mutations that prevent induction of transcription, mutations that eliminate ethanolamine ammonia-lyase, and mutations that eliminate the second enzyme in the degradation pathway, acetaldehyde dehydrogenase (27). The genetic map also includes mutations that do not affect either of these enzyme activities but nevertheless cause an inability to use ethanolamine. The existence of these mutants suggests that additional functions necessary for ethanolamine utilization are encoded in the eut region.

We describe here results of complementation tests performed to determine the number of genes encoded in the eut operon. Six genes were defined, including two for ethanolamine ammonia-lyase (eutB and eutC), one for acetaldehyde dehydrogenase (eutE), and one whose activity is required for induction of transcription of the eut operon (eutR). One of the newly defined genes (eutA) was shown to be required for ethanolamine utilization only when CN-B12 or HO-B12 were the precursors of the Ado-B12 cofactor of ethanolamine ammonia-lyase. Ethanolamine uptake experiments suggest that no ethanolamine permease is encoded in the eut operon. The F' plasmid used in the complementation tests was constructed by a transpositional method that allows one to place any region of the chromosome on an F plasmid.

**MATERIALS AND METHODS**

**Bacterial strains and transposons.** All strains used in this study are derivatives of *S. typhimurium* LT2 (Table 1). The eut mutations used in the complementation tests are listed only by their allele numbers (see below); isolation and genetic mapping of these mutations was described previously (27). Two transposition-defective derivatives of the specialized transducing bacteriophage Mu d1(Amp' lac cts) of Casabadian and Cohen (6) were used. The derivatives, MudI734 Kan' (7) and Mu d1-8 Amp' (21) are referred to as MudJ and MudA, respectively; each can form lac operon fusions upon insertion. A transposition-defective derivative of Tn10, Tn10dcam (14), was used.

**Media and growth conditions.** The complex medium was nutrient broth (0.8%; Difco Laboratories) with NaCl (0.5%). The minimal medium was the E medium of Vogel and Bonner (32) with glucose (0.2%). The carbon-free minimal medium was NCE (12), and the carbon- and nitrogen-free minimal medium was NCN (26). Ethanolamine hydrochloride (0.2%; Aldrich Chemical Co.) 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 sources in NCN medium (0.2%, 1.2-Propanediol (0.4%; Aldrich) was used as the carbon source in NCE medium or in NCN medium with ethanolamine as the nitrogen source. CN-B12, HO-B12, and Ado-B12 (all from Sigma Chemical Co.) were used as exogenous B12 sources (0.1 μg/ml). Medium containing Ado-B12 was protected from exposure to light during preparation and use. Amino acids were added to minimal media as required at the concentrations previously recommended (12). Cystine (0.058 mM) was added to nutrient broth used for growth of cys auxotrophs. Antibiotics were used at the following concentrations (micrograms per milliliter) in mineral and complex media, respectively: ampicillin, 15 and 30; chloramphenicol, 5 and 20; kanamycin, 125 and 50; and tetracycline, 10 and 20. Solid medium contained agar (1.5%; Difco) or, when it was necessary to score growth on medium containing ethanolamine as the sole nitrogen source, Noble agar (1.5%; Difco). Cells were grown aerobically at 37°C.

**Genetic techniques.** All transductional crosses were performed with the high-frequency, generalized transducing phage mutant P22 HT105/1 int-201 (29) as previously described (27). Transductants were purified and made phage-free by streaking for single colonies on nonselective green indicator plates (8).

All conjugational crosses were performed by mixing liquid cultures (0.05 ml each) of the donor and recipient strains on one side of a petri dish containing selective medium. The plate was incubated at 37°C for 3 to 6 h, and the cell mixture was then streaked to obtain single exconjugant colonies after further incubation.

**Placement of the eut region on an F plasmid.** A composite transposon, consisting of the eut operon and two flanking Tn10 insertions, was used to introduce the eut genes onto the F' T80 his + plasmid by transposase-mediated transposition. The composite transposon was constructed on the chromosome in two steps. A Tn10 insertion located adjacent to the promoter proximal end of the eut operon (zfa-3649::Tn10) was transduced into the eut-cys deletion mutant TT11701 by selecting for Tet + recombinants. A transductant containing

**TABLE 1. Bacterial strains used**

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<tr>
<th>Strain</th>
<th>Genotype LT2</th>
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<tr>
<td>LT2</td>
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<td>TR25</td>
<td>his-712 ser-821 arg-501/T' T80</td>
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<td>metE205 ara-9</td>
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<td>his-2236</td>
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<td>TT11289</td>
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<tr>
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<tr>
<td>TT14771</td>
<td>metE205 ara-9 eutA114 zfa-3648::Tn10</td>
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* All strains were constructed for this study or were obtained from the laboratory collection.
both the Tn10 insertion and the deletion mutation (Eut− Cys− Tet+) was kept (TT14066). A second Tn10 insertion (zfa-3647::Tn10) is located adjacent to the promoter-distal end of the eut operon and within the region removed by the eut-cys deletion; this insertion was transduced into strain TT14066 by selecting for Cys+ recombinants. Both Eut+ and Eut− transductants were recovered. The Eut− transductants contain a deletion of eut generated when the donor and recipient Tn10 elements recombine. The Eut+ transductants that carry two copies of Tn10 flanking eut were identified by instability of the Eut+ phenotype due to loss of eut by recombination between the chromosomal Tn10 elements. The unstable Eut+ transductants carry the composite transposon shown in Fig. 2. The strain containing the composite transposon (TT14068) was maintained on medium containing ethanolamine as the sole nitrogen source to select against loss of eut by recombination between the flanking Tn10 elements. Strain TT14071 is a His− Kan+ derivative of strain TT14068 made by transduction of the hisD9953::MuD1 insertion from strain TT10286. The F′ Tn14071::Tn10 plasmid from strain TR35 was introduced into strain TT14071 by conjugation, selecting for a His+ exconjugant (TT14074).

Transposition of the composite transposon into the plasmid F′ T80 his+ was detected by conjugational mating of strain TT14074 with a Eut− Cys− His− Amp+ recipient (TT14076). Selection was made for exconjugants that were His+ (specified by plasmid F′ T80), Eut+ (conferring an insertion of the composite transposon within plasmid F′ T80), and Amp+ (to counter-select the donor). The cross was done by spreading 0.5 ml of an overnight culture of donor cells with 0.5 ml of recipient cells on NCE medium containing ethanolamine, CN-B12, cystine, and ampicillin. Control crosses with the same donor-recipient pair were done without selection for Eut− on E medium containing glucose, tetracycline, ampicillin, and cystine to select for simple insertions of Tn10 in the F′ T80 plasmid. Selection for Tet+ yielded about 1,000-fold more exconjugants than selection for Eut+, suggesting that the individual Tn10 elements transpose substantially more frequently than the composite unit including the eut operon. One F′ His+ Eut+ Tet+ plasmid (described in Results and designated F′ 606) was used for complementation tests.

**Complementation tests.** The eut point mutations to be used in complementation tests were placed on F′ 606 by cotransduction with a Tn10 insertion near the eut region (zfa-3646::Tn10 for eut-1 through eut-55 or zfa-3648::Tn10 for eut-56 through eut-138). The eut::MuD1 and eut::Tn10 insertions were placed on F′ 606 in transductional crosses by selecting for Kan+ or Tet+ recombinants. The recipient used was strain TT14078 or strain TT14082. In these crosses, the eut mutations and adjacent Tn10 insertion are inherited only by recombination with the plasmid eut region because the recipient chromosome carries a deletion of eut and cys4 that is rarely repaired by P22 transduction. Retention of the chromosomal deletion by all Eut− recombinants was confirmed by verifying their requirements for cystine. The isolation of the eut mutants used in this study and the construction of a genetic map of the eut region that includes the mutations have been described previously (27).

To construct recipient strains for the complementation tests, eut point mutations were introduced into strain TR7020 (his-2236) by transduction using one of the adjacent Tn10 insertions as the selective marker. The eut::MuD1 and eut::Tn10 insertions were introduced into strain TR7020 in transductional crosses selecting Kan+ or Tet+ recombinants. To make these strains recombination deficient, P22 grown on a strain containing a Tn10 insertions near the recA1 mutation (TT11289) was used to transduce the Eut− His+ strains to Cam+. The recA recombinants were identified by UV light sensitivity.

To construct diploids for the complementation tests, each eut− F′ plasmid was introduced into the Eut+ His+ RecA− recipients by conjugational crosses selecting inheritance of the plasmid his+ marker. Donor strains were grown overnight in minimal glucose medium containing cystine, and recipient strains were grown overnight in minimal glucose medium with histidine. The donor and recipient strains were plated on minimal glucose plates, and after growth, four isolated colonies from each cross were picked and patched on minimal glucose plates. These plates were incubated 6 to 12 h and used as masters to make replica prints to medium containing ethanolamine as the sole carbon and nitrogen source. Complementation was scored as positive if significant growth of the four patches occurred after 3 to 5 days of incubation.

**Ethanolamine uptake assays.** Cells to be used in the uptake assays were grown with vigorous agitation to an A650 of 0.4 to 0.6 in NCE medium containing glycerol as the carbon source. The medium was supplemented with ethanolamine and CN-B12 (to induce eut operon expression) unless indicated otherwise. The cells were washed twice by centrifugation and suspension in NCE medium; following the third centrifugation, cells were suspended in NCE medium containing 0.1% glucose to obtain an A650 of 0.66 (about 10⁶ cells per ml) and kept on ice. To measure ethanolamine uptake, a 0.5-ml sample of the cell suspension was preincubated for 3 min at 25°C, and [14C]ethanolamine (53 μCi/ml; Amer- sham Corp.) was then added to a final concentration of 10 μM. Samples of the reaction mixture were removed after 15, 30, 45, 60, and 120 s of incubation at 25°C; cells from each sample were immediately collected on membrane filters (HA, 0.45-μm pore size; Millipore Corp.) and washed twice with 4 ml of NCE medium containing 0.2% unlabeled ethano- lamine. The filters were dried with a heat lamp, and the amount of labeled ethanolamine taken up was determined by scintillation counting.

**RESULTS**

**Construction of an F′ plasmid containing the eut operon.** F′ plasmids that include the eut operon were generated by transposition of the chromosomal eut region of S. typhimurium to an existing F′ plasmid. Transposition of the eut region was achieved by constructing a composite transposon consisting of the eut+ genes flanked by Tn10 elements (Fig. 2). The transposase encoded by the IS10 elements of Tn10 can catalyze transposition of the composite element (Tn10 eut+ Tn10) to a new site. The behavior of a similar com- posite transposon consisting of the malK gene from E. coli and flanking Tn5 elements has been previously described (19).

Figure 2 presents the various eut-containing subsets of the composite unit that should be able to transpose; one subset includes only the eut+ operon and an IS10 element from each of the flanking Tn10 transposons. The plasmid F′ T80 his+ was provided as a target for insertion by the composite transposon. This plasmid was chosen because of its ability to transfer and its low copy number. Plasmids that acquired an insertion of the composite transposon were identified by conjugational crosses with an Eut− His+ recipient by selecting for Eut+ His+ exconjugants. Both Tet+ Eut+ His+ and Tet− Eut+ His+ plasmids were isolated as expected from the transposable subsets of the composite element (Fig. 2).
FIG. 2. The composite transposon consisting of the eut operon and two flanking Tn10 insertions. New insertions of the composite transposon which include eut also include either Tn10 or IS10 at each end. The four possible transposable units are shown. Both Tn10 elements are in the B orientation as defined relative to other Tn10 insertions in reference 10. The orientation of an A Tn10 insertion (hisG9424::Tn10) relative to the chromosome is known (17), making it possible to define the chromosomal orientation of zfa-3647::Tn10 and zfa-3649::Tn10. The zfa-3647::Tn10 insertion (shown on the left) is located between eut and purC, while the zfa-3649::Tn10 insertion (shown on the right) is located at the opposite end of the eut operon near cysA. Transcription of eut is from left to right.

One of the Tet plasmids (F' 606) was used in all subsequent experiments. The eut' and his' markers of F' 606 were inherited simultaneously in all subsequent conjugal crosses. This plasmid is quite stable; about 10 generations of nonselective growth yielded Eut' His' segregants at a frequency of $10^{-3}$ and no Eut' His' segregants were found (frequency $<10^{-7}$). The stability of the eut region is probably due to the fact that the repeated flanking IS10 elements are in inverse orientation and thus do not recombine to eliminate the eut operon.

Six complementation groups included in the eut locus. The complementation behavior of eut mutations was tested in strains made diploid for eut by introduction of F' 606. The eut mutations were placed on F' 606 by transductional crosses, and the resulting eut plasmids were introduced into various eut his recA recipients by selection for inheritance of the his' marker of the plasmid (see Materials and Methods). Complementation was scored by testing the exconjugants for growth on medium containing ethanolamine as the sole source of carbol and nitrogen.

The results of the complementation tests are shown in Fig. 3. Each mutation was tested in both the chromosomal and plasmid positions. The 40 mutations tested defined six complementation groups. Each gene is defined by several point mutations which do not complement each other but which do complement point mutations in the other genes. In Fig. 3, mutations are presented in order (horizontally and vertically) according to their positions in the genetic map described previously (27). It can be seen that mutations inferred to affect the same complementation group map in a contiguous cluster. Point mutations and Tn10 insertion mutations that seem to have a polar effect on expression of downstream genes are also included (see below). Occasionally, complementation behavior was affected by whether the alleles being tested were on the plasmid or on the chromosome. Possible explanations for this are presented in the discussion.

Some of the mutations used in the complementation tests were previously tested for their effects on the in vitro activity of the two enzymes required for ethanolamine utilization, ethanolamine ammonia-lyase and acetaldehyde dehydrogenase (27). The gene designation inferred from the complementation tests and a summary of enzyme activities is shown in Fig. 4. These results indicate that the eutB and eutC genes specify ethanolamine ammonia-lyase and that the eutE gene specifies acetaldehyde dehydrogenase. All of the mutations previously shown to cause inability to express the entire eut operon (27) fell into a single complementation group, eutR; we presume that this gene encodes a positive regulatory protein. Results suggesting the role of the eutA gene product are presented later. The role of the eutD gene product remains undefined, except that it is required only for use of ethanolamine as a carbon source.

Transcriptional polarity and independent expression of eutR. Several point mutations and Tn10 insertion mutations failed to complement mutations inferred to be in downstream genes (examples are eutD46::Tn10 and eutA68 in Fig. 3). The orientation of the polar effects is consistent with the direction of transcription of the eut operon (from left to right in Fig. 3 and 4) which was determined previously by using eut-lac operon fusions (27). Nevertheless, none of the mutations in the eutD, eutE, eutA, eutB, or eutC genes, including the polar mutations mentioned above and the eutC159::MudJ insertion, exerted a polar effect sufficient to prevent complementation with eutR mutations. This suggests that the eutR gene can be transcribed from a promoter located downstream of the eutC159::MudJ insertion site. In fact, transcription of the eutR gene occurs from both the
FIG. 4. Gene-function relationships. The name of each complementation group and representative mutations in each group are shown at the top. The mutations are listed in their map order and transcription is from left to right. Below each complementation group is a summary of the phenotype and in vitro enzyme activities of strains containing the listed mutations. C* indicates an ability to use ethanolamine as a carbon source (aerobically with CN-B12); N* indicates an ability to use ethanolamine as a nitrogen source (aerobically with CN-B12). For in vitro ethanolamine ammonia-lyase activity (EAL) and in vitro acetalddehyde dehydrogenase activity (ACDH), a + indicates an in vitro enzyme activity of 30 to 100% of that present in wild-type strains and a − indicates an in vitro enzyme activity of less than 1% of that present in wild-type strains. The enzyme assay data has been previously published (27). An exceptional mutant, eutE91, has in vitro ethanolamine ammonia-lyase activity of only 7% of that present in wild-type strains. This mutation may have a slight polar effect on transcription of the downstream genes for ethanolamine ammonia-lyase (eutB and eutC).

regulated promoter for the operon and from the promoter located downstream of the eutC159::MudJ insertion site (unpublished results).

Utilization of Ado-B12 precursors. The first enzyme in ethanolamine utilization, ethanolamine ammonia-lyase, requires the cofactor Ado-B12 for activity (4, 28). This cofactor is synthesized de novo during anaerobic growth. The B12 biosynthetic pathway is outlined in Fig. 1. To permit ethanolamine utilization during aerobic growth, B12 must be obtained from the growth medium. Exogenous B12 is usually supplied as CN-B12 or HO-B12, neither of which is active as a cofactor until converted to Ado-B12 in several enzymatic steps. In fact, the inactive precursor forms of B12 are potent inhibitors of ethanolamine ammonia-lyase in vitro (3). S. typhimurium can convert HO-B12 or CN-B12 to the Ado-B12 required by ethanolamine ammonia-lyase (Fig. 1).

Mutants defective in the eutA gene can use ethanolamine as the nitrogen source when Ado-B12 is provided but not when HO-B12 or CN-B12 is provided. This was determined by testing the abilities of various eut mutants to use ethanolamine as a carbon or nitrogen source when Ado-B12 was provided (Table 2). These results suggest a role for the eutA gene product in the use of HO-B12 and CN-B12 as precursors of Ado-B12. It should be noted that eutA mutants could not use ethanolamine as a carbon source even when Ado-B12 was provided (Table 2). This may be due to insufficient transport of intact Ado-B12 (see Discussion for alternative explanations).

Strains containing mutations in the eutB, eutC, and eutR genes were unable to degrade ethanolamine for use as either a carbon or a nitrogen source even when Ado-B12 was provided (Table 2). Strains containing mutations in the eutD and eutE genes fail to use ethanolamine as a carbon source but are able to use it as a nitrogen source (with any form of B12 provided). Since eutD and eutE mutants are able to derive ammonia (but not carbon) from ethanolamine, they must retain the ability to transport ethanolamine and synthesize the Ado-B12 required as a cofactor by ethanolamine ammonia-lyase; as expected, the growth defect of these mutants was not corrected by providing Ado-B12 (Table 2).

The eutA mutants were able to use HO-B12 or CN-B12 for two other B12-dependent enzymes in S. typhimurium. One of these enzymes, a B12-dependent methyltransferase, is one of the two enzymes that can methylate homocysteine to form methionine (16). The function of the B12-dependent enzyme was tested in a metE mutant which lacks the alternative B12-independent methyltransferase enzyme (Table 2). The metE eutA double mutant was unable to grow on minimal medium but did grow on medium supplemented only with HO-B12 or CN-B12, indicating that eutA mutants continued to transport B12 and synthesize the methyl-B12 cofactor needed by the B12-dependent methyltransferase. The possibility remains that HO-B12 and CN-B12 transport is partially impaired in eutA mutants.

The eutA mutants retain the ability to synthesize Ado-B12 from HO-B12 or CN-B12 precursors. This was evident because the Ado-B12 requirement of another Ado-B12-dependent enzyme, propanediol dehydratase, was satisfied by

<table>
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<th>Relevant genotype</th>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>B12-dependent function tested</th>
<th>Growth of strain aerobically on medium containing the indicated form of B12</th>
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<td>eutD101 or eutE55</td>
<td>Glycerol</td>
<td>EA</td>
<td>EAL (nitrogen)</td>
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<td>+</td>
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<td>EA</td>
<td>NH₄</td>
<td>EAL (carbon)</td>
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<tr>
<td>metE205</td>
<td>Glucose</td>
<td>NH₄</td>
<td>MT (methionine)</td>
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<td>+</td>
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<tr>
<td>metE205, eutA114</td>
<td>Glucose</td>
<td>NH₄</td>
<td>MT (methionine)</td>
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</tr>
<tr>
<td>Wild type</td>
<td>PD</td>
<td>NH₄</td>
<td>PDD (carbon)</td>
<td>−</td>
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<tr>
<td>eutA114</td>
<td>PD</td>
<td>NH₄</td>
<td>PDD (carbon)</td>
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<td>EA</td>
<td>EAL (nitrogen) and PDD (carbon)</td>
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<td>EA</td>
<td>EAL (nitrogen) and PDD (carbon)</td>
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</table>

* EA, ethanolamine; EAL, ethanolamine ammonia-lyase; MT, 5-methyltetrahydrofolate-homocysteine transmethylase; PD, propanediol; PDD, propanediol dehydratase.
* All forms of B₁₂ were supplied at 0.1 μg/ml.
* The growth requirement satisfied by the B₁₂-dependent enzyme is indicated in parentheses.

TABLE 2. Abilities of eut mutants to use Ado-B₁₂ precursors

To satisfy its nutritional requirements, S. typhimurium requires the utilization of several precursors of Ado-B₁₂, which is synthesized de novo during anaerobic growth. These precursors are derived from ethanolamine, which is a nitrogen source as well as a carbon source.
HO-B12 or CN-B12 in eutA mutants. The Ado-B12-dependent propanediol dehydratase has only recently been identified in *S. typhimurium* (R. Jeter, personal communication). The propanediol utilization pathway enabled both wild-type and eutA mutant strains to grow on medium containing propanediol as the sole carbon source and ammonia as the sole nitrogen source when HO-B12 or CN-B12 was provided (Table 2). It is possible that alternative enzymes which convert HO-B12 or CN-B12 to Ado-B12 are induced only during growth on propanediol. To determine if a function induced during growth on propanediol can substitute for the eutA gene product, the eutA mutants were tested for the ability to grow on medium containing propanediol as the sole carbon source (to induce this pathway) and ethanolamine as the sole nitrogen source (to test whether sufficient Ado-B12 was made) (Table 2). The eutA mutants were unable to grow on this medium, indicating that no substitute for the eutA gene product appeared during growth on propanediol.

The above observations may be explained by the fact that ethanolamine ammonia-lyase is acutely sensitive to inhibition by CN-B12 and HO-B12. The eutA gene product may act to prevent this inhibition in vivo. To test this possibility, the ability of a eutA mutant to use a mixture of CN-B12 and Ado-B12 for growth on ethanolamine as the nitrogen source was checked. Ado-B12-dependent growth was completely inhibited by an equal concentration of either HO-B12 or CN-B12 in the medium; this inhibition was not seen for eutA− cells, which use ethanolamine with either or both of these B12 forms at the same concentration. This suggests that the function of the eutA gene product may be to remove inhibitory forms of B12 or to allow ethanolamine ammonia-lyase to discriminate between these B12 forms.

**The eut operon does not appear to include an ethanolamine permease.** The rate of ethanolamine uptake by eut mutants was examined to determine whether an ethanolamine permease is encoded in the eut operon. The uptake assays were performed on cells that were grown in glycerol-ammonia medium containing ethanolamine and CN-B12 to induce expression of the eut operon. The data in Fig. 5 show that strain LT2 accumulated ethanolamine at a high and nearly constant rate throughout the 4-min assay. Accumulation of ethanolamine in point mutants defective in any of the individual *eut* genes occurred at lower rates. It appears that uptake of ethanolamine at a high rate requires concurrent degradation of ethanolamine by both ethanolamine ammonia-lyase and acetaldehyde dehydrogenase. Ethanolamine uptake did not occur when the proton gradient was abolished by addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone. Ethanolamine uptake did not occur when the proton gradient was abolished by addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone to the cells before the assay began (Fig. 5).

Although transport of ethanolamine was reduced in all of the individual *eut* mutants, a significant residual level of transport remained. This residual level of transport was blocked by carbonyl cyanide m-chlorophenylhydrazone, suggesting that the proton gradient may contribute to transport. Since all mutations reduced transport to a similar extent, we presume that all exert their effect by impairing degradation and none of the six known genes in the operon encode a component of the transport mechanism.

To test more directly for the presence of a permease gene in the *eut* operon, we compared two strains, both blocked in ethanolamine degradation. One carried a deletion of the entire operon (*eut*-237); the other carried a point mutation in one of the genes for ethanolamine ammonia-lyase (*eutC110*). If an ethanolamine permease is encoded in the *eut* region, it should contribute to the lyase-independent ethanolamine uptake activity in the *eutC* point mutant but not in the deletion mutant. The uptake rates observed for the two strains were identical (Fig. 6), suggesting that the deletion mutant lacks only the ability to degrade ethanolamine. The ethanolamine uptake occurred only when the proton gradient was left intact; this level was present in wild-type cells grown in the absence of ethanolamine and B12 (Fig. 6). For the assays shown in Fig. 6, 20 μM ethanolamine was present. When the concentration of ethanolamine was increased to 100 μM, the uptake rates increased for both strains, but the two strains could still not be distinguished (data not shown).
DISCUSSION

An F' plasmid containing the eut region was constructed and used to perform complementation tests that defined six complementation groups in the eut operon. A summary of representative mutations in each complementation group and the enzymatic or growth defect caused by the mutations is shown in Fig. 4. Mutations in the eutR gene prevent expression of the entire eut operon; strains containing a eutR mutation lack all of the enzymatic activities encoded in eut and fail to express eut-lac operon fusions located promoter proximal to the eutR gene (27). Mutations in the eutB and eutC complementation groups affect in vitro ethanolamine ammonia-lyase activity. Two complementation groups for ethanolamine ammonia-lyase were expected because this enzyme isolated from Clostridium sp. and E. coli is composed of two subunit types (3, 34). Mutations in the eutE complementation group affect in vitro acetaldehyde dehydrogenase activity; this enzyme is composed of a single subunit type in E. coli (24). The function of the eutD gene product is not yet known; these mutants are able to use ethanolamine as a nitrogen but not a carbon source.

In the complementation tests, several point and insertion mutations exhibited a polar effect on the expression of downstream genes. Nevertheless, the function of the most promoter-distal gene in the operon (eutB) was not eliminated by upstream mutations (including insertion mutations), indicating that eutR can be expressed from its own promoter. In a subsequent paper, we will show that eutR can also be expressed from the main promoter for the eutDEABC operon and that the independent promoter for eutR is a constitutive promoter within the operon.

Many mutations that appeared to be nonpolar since they complemented many downstream mutations still failed to complement particular alleles of downstream genes. In these cases, complementation was often restored when the chromosomal and the F' plasmid loci of the two mutations were switched. Perhaps the relative concentration of each eut gene product is important for efficient utilization of ethanolamine. This might reflect the need for an enzyme complex of the enzymes for ethanolamine degradation or a toxic effect of accumulated acetaldehyde.

The eutA gene product is required for use of ethanolamine in the presence of exogenous HO-B₁₂ or CN-B₁₂. The eutA mutants could use exogenously supplied Ado-B₁₂ to fulfill the cofactor requirement for growth on ethanolamine as the sole nitrogen source (but not as the sole carbon source) (Table 2). Several results suggest that the growth defect of eutA mutants is not caused by a complete lack of Ado-B₁₂ synthesis but by inhibition of ethanolamine ammonia-lyase by accumulated HO-B₁₂ or CN-B₁₂. First, the eutA mutants could still convert HO-B₁₂ or CN-B₁₂ to Ado-B₁₂ to fulfill the cofactor requirement of the Ado-B₁₂-dependent enzyme D1-1,2-propanediol dehydratase. The Ado-B₁₂ inferred to be available in eutA mutants during growth on propanediol as the carbon source when HO-B₁₂ or CN-B₁₂ was provided exogenously did not permit the simultaneous use of ethanolamine as the nitrogen source. A more direct indication that accumulated HO-B₁₂ or CN-B₁₂ might inhibit ethanolamine ammonia-lyase is the fact that eutA mutants failed to use Ado-B₁₂ if HO-B₁₂ or CN-B₁₂ was provided simultaneously. The eutA gene product may serve to protect ethanolamine ammonia-lyase from inactivation by preventing accumulation of a high concentration of inhibitory B₁₂ forms in the cytoplasm. This could be accomplished by increasing the rate of adenylation, by slowing entry of HO-B₁₂ or CN-B₁₂ into the cytoplasm, or by inactivating internal forms of B₁₂ other than Ado-B₁₂. Alternatively, the eutA gene product could interact with ethanolamine ammonia-lyase directly to protect it from inactivation by the toxic B₁₂ forms. The ethanolamine ammonia-lyase enzyme itself seems to be functional in eutA mutants because cell extracts of eutA mutants retain normal ethanolamine ammonia-lyase activity in an assay dependent on added Ado-B₁₂ (27).

Exogenously supplied Ado-B₁₂ allowed the eutA mutants to use ethanolamine as the sole nitrogen source but not as the sole carbon source (Table 2). During the course of catalysis by ethanolamine ammonia-lyase, the carbon-cobalt bond of Ado-B₁₂ undergoes reversible cleavage and reformation (1). It is thought that the carbon-cobalt bond occasionally fails to reform correctly, generating inactive (inhibitory) forms of B₁₂ as a by-product of catalysis (2, 20). We suggest that these inactive forms of B₁₂ accumulate in a eutA mutant during the large number of reaction cycles required to use ethanolamine as the sole carbon and energy source. Thus, the role for the eutA function may be to regenerate Ado-B₁₂ and thereby protect ethanolamine ammonia-lyase from inhibition by its own accumulated by-products. Such an activity might also protect the enzyme from inhibition by cytoplasmic HO-B₁₂ or CN-B₁₂.

An ethanolamine permease does not seem to be encoded in the eut operon since a deletion mutant that lacks the entire eut operon still transported ethanolamine at the same rate as a strain that contains only a point mutation in one of the genes for ethanolamine ammonia-lyase. It is possible that the eut operon encodes an ethanolamine permease whose activity is not detectable under our assay conditions or whose activity requires the presence of active ethanolamine ammonia-lyase enzyme. All uptake measured was inhibited by pretreatment of cells with a drug that abolishes the proton gradient, suggesting that ethanolamine uptake is mediated by an active transport system. So far, no Eut+ mutant unlinked to the eut operon has been isolated that lacks ethanolamine uptake activity (D. Roof, unpublished). If several permeases act on ethanolamine (as is the case for some other growth substrates [11]) or if ethanolamine diffuses through the membrane, our failure to recover permease-deficient Eut− mutants would be explained.

The method used here to construct an F' plasmid containing the eut operon can easily be applied to other bacterial genes. The main advantage of the method is that a large (40 kilobases or more) genetically predetermined region can be placed on a low-copy-number F' plasmid. Plasmids such as these are difficult to construct by other methods.

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LITERATURE CITED
